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From the Institute of Physiology, University of Uppsala, Uppsala, Sweden

An Analysis of the Current-Voltage Relationship in Excitable Nitella Cells

By

TORSTEN TEORELL

Received 15 February 1961

Abstract

TEORELL, T. *An analysis of the current-voltage relationship in excitable Nitella cells.* Acta physiol. scand. 1961. *53.* 1—6. — Experiments were performed on cells of the fresh water algae *Nitella* using "triangular waves" of varying frequencies, *i. e.* depolarizing-hyperpolarizing currents with varying, linear slopes. With very low frequencies one obtains "steady state rectification" curves, but with intermediate frequencies instability phenomena are revealed. The experimental findings of "dynamic current-voltage" characteristics are compared with the theoretical results, which can be predicted from the author's "electrohydraulic" hypothesis for excitability phenomena. It is suggested that electro-osmotic processes, arising from presence of membrane fixed charges, may play a part in the excitability of *Nitella.*

In recent years it has become common to characterize the excitability properties of nerves and other tissues by measuring the changes in the transmembrane potential after application of hyperpolarizing or depolarizing currents. The results are usually plotted as current versus resulting voltage. Such a current-voltage relationship is also denoted "rectification curve", because the slopes to the curve define the tissue (slope) conductance (COLE and CURTIS 1941). The usual finding is that the rectification curve indicates a marked increase in conductance during depolarisation. For the single nerve fiber it is well known that there can be an apparent change between resting conductance and spike conductance of the order of 1 : 200. The nature of this marked change has been discussed in various terms as "delayed rectification etc." For a number of years the author has attempted to analyse the rectification

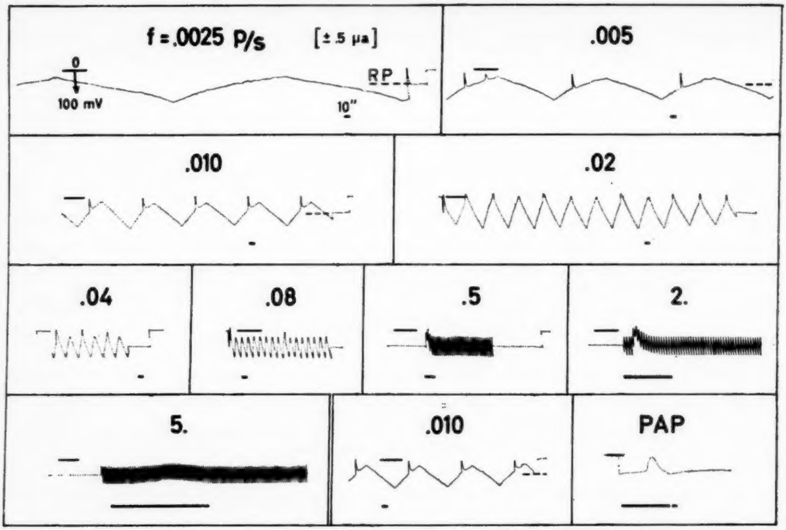

Fig. 1. A *Nitella* cell subject to triangular current waves of varying frequencies. Peak current amplitudes $\pm$ 0.5 μA. Horizontal bars indicate zero potential. Dashed bars, marked *RP*, indicate resting potential. Time bars represent 10 sec. *PAP* shows a propagated action potential following the experiment.

curves in terms of an "electrohydraulic" theory in which electro-osmosis has a prominent part (Teorell 1958 a, 1959 a, b, c, 1960). In a paper by Teorell (1958 b) an attempt was made to investigate the rectification in a plant cell *(Nitella)* in relation to the electro-osmosis. The results indicated at least a formal validity of the electrohydraulic hypothesis. Since then we have performed numerous investigations on the *Nitella* with various methods in order to characterize the excitability mechanism. Especially useful we have found the application of triangular current waves of different frequencies. With very slow waves (of a frequency of the order of 0.002 p/s) it was possible to obtain a current-voltage relation curve, which was smooth and almost without hysteresis and without any appearance of "action potential" responses (the resulting plot is in shape similar to that marked *Q* in Fig. 2 b). In the meantime Findlay (1959) has published a current-voltage relation for the *Nitella*, which has a similar shape, although without attempts to a closer analysis of its nature.

Experiments on the current-voltage characteristics of the Nitella. The steady state method of using depolarisation and hyperpolarisation currents as employed by for instance Findlay, gives meager information about the possible intrinsic nature of the resulting rectification curve. More information could be ob-

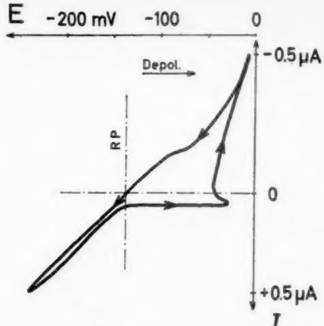

Fig. 2 a.

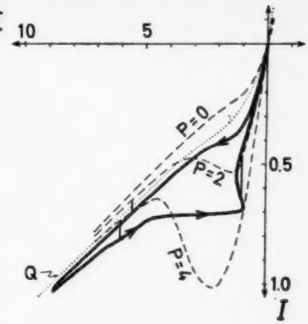

Fig. 2 b.

Fig. 2 a. A current-voltage record of *Nitella* with a triangle wave of f = 0.010 p/s. RP = resting potential (before addition of NaCl).

Fig. 2 b. A reconstructed current-voltage relation according to the "electrohydraulic" theory. See text for explanation.

tained by investigation of the *non*-steady state behaviour, which reveals interesting details of the dynamic properties of the excitable system. We have therefore employed "triangular" depolarisation-hyperpolarisation current waves of a frequency, which discloses otherwise "hidden" instability phenomena.

Methods

The experimental technique was essentially the same as described previously (Teorell 1958 b), with the exception that the square wave generator was substituted by a triangular wave generator. The frequency was varied in the range of 0.0025 p/s to 5.0 p/s. The peak current values were + 0.5 and — 0.5 microamps corresponding to a current density of about 5 microamps/cm^2. One current electrode was placed in a pool of pond water and another in 0.1 M KCl, *i. e.* the essential changes observed took place under the pond water electrode.

Results

A typical result is depicted in Fig. 1 and shows that potential spikes are elicited at an intermediate frequency range of about f = 0.01 p/s. The nonlinear voltage response is especially evident at f = 0.0025 and 0.005 p/s. An interesting feature is also offered by the very high frequency response at 0.5 and 2.0 p/s; however, these results will not be discussed in this paper.

The next step in the experimental analysis was to select the "dynamic" f = 0.01 condition and by means of a X-Y-plotter obtain the current-voltage relationship. Such a result is depicted in Fig. 2 a. In this particular experiment, however, some NaCl was added to the pond water in order to obtain an outside electrolyte concentration suitable for comparison with the theoretical analysis to follow (0.02 molarity of NaCl was used). This has also the added advantage of making the cell more excitable or even inducing spontaneous

firing. The nature of this NaCl-stimulation will be discussed in a forthcoming paper. The *I-E* characteristic of Fig. 2 a is by no means a single curve; *i. e.* it displays marked "hysteresis". An abrupt change takes place during the depolarisation phase, close to the resting potential a sudden "flip over" is noticed. During the repolarizing return trace there is also a sudden brief transition in the voltage.

A theoretical analysis of the dynamic current-voltage relation. The following interpretation of the observed events is based essentially on the same concepts emerging from the electro-osmotic membrane oscillator, which were used already in the previous "square wave analysis" of the *Nitella* (TEORELL 1958 b). However, an additional process allowing for membrane leakage has been added as described in the formalism given by TEORELL (1959 c, 1960).

The numerical solution was carried out by the analog computer technique according to the principles described by TEORELL (1960). The results are obtained directly as graphs (*e. g.* Fig. 2 b). The following numerical values were used in the machine solution of the electrohydraulic excitability equations: s (hydraulic permeability coefficient) $= 1.0$; σ (leakage coefficient) $= 1.6$; l (electro-osmotic permeability coefficient) $= 1.5$ and further coefficients were set as $k = 40$, $q = 0.03$ and an additional V_E decrement of 10 units was added to allow for the effect on the water drag exerted by the external salt. The remaining parameters were exactly the same as described by TEORELL (1959 b, 1960). The machine frequency of the employed triangle wave was 0.5 p/s. The results obtained are given in arbitrary machine units and the zero current is displaced to the origin. The curves for constant ("clamped") hydrostatic pressure, marked P (dashed lines), as well as the steady state rectification curve, marked Q (dotted line), were obtained by appropriate machine operations.

The theoretical reconstruction result is depicted in Fig. 2 b. The dynamic path (the solid line with direction arrows) clearly shows a marked similarity with the experimental *Nitella* result shown in Fig. 2 a. The similarity appears to be so close in its essential features, that a detailed discussion of its significance seems worth while.

Discussions on the nature of the current-voltage characteristic. — In the electrohydraulic excitability theory the state variables are the transmembrane potential (E), the membrane current (I) and the hydrostatic pressure difference (P) across the excitable membrane. The pressure factor is the resultant of the superposition of electrochemical and electro-osmotic driving forces (full discussions are given by TEORELL 1959 b).

According to this hypothesis an action potential, or a continuous change in the polarisation state created by current flow, should produce concomitant changes in pressure (and water flow). A full presentation of the interrelations between these three "state variables" is most rationally obtained by a three-dimensional "phase space" representation. Such an *I-E-P* representation can be found in another paper by the author (1961). The two-dimensional *I-E* "phase plot" is accordingly somewhat deficient as it does not immediately reveal the influence of the pressure variable. However, in the Fig. 2 b some

steady state *I-E* graphs are given for a few constant *P*-values (as dashed lines). These *P* "clamped" graphs should be visualized in planes parallel with the page sheets, as a third dimension perpendicular to the paper plane. The two *P*-clamps with $P > 0$ have a marked N-shaped course and are of a so called "dynatron" character (cf. FRANK 1956; FITZHUGH 1960), a concept often used in modern neurophysiology analysis of the "bistability" properties of, for instance, the nerve axon (cf. SPYROPOULOS and TASAKI 1960). It is characteristic of the dynatron-curves that they have a negative slope in the intermediate part, which signifies negative conductance. On the other hand, it is known from oscillation theory, that the presence of negative conductances may lead to instability phenomena and oscillations. A closer analysis of the dynamic current-voltage path in Fig. 2 b reveals that it extends in the three dimensions (in the figure the brief vertical lines connect the pressure graphs with the corresponding points of the path). The sudden transitions, or flip overs, of the *I-E* path belong actually to the instability domains of numerous dynatron curves. It can also be shown that the spontaneous, or induced, action potentials can be described in terms of a three-dimensional variation in the current-voltage-pressure space.

It may be added, that the computer analysis also quite faithfully predicts the frequency dependence of the triangular wave responses of the *Nitella* as presented in Fig. 1. In particular it should be pointed out that the very low frequency, *i. e.* the steady state *I-E* response, as depicted by the dotted line marked *Q* in Fig. 2 b, is similar to the one published by FINDLAY and also obtained by us.

The demonstration that the dynamic *I-E*-characteristic is a projection in a two-dimensional plane obtained from a three-dimensional *I-E-P*-space, of course, does not prove that the pressure variable is of importance in the living *Nitella* cell. But in any case, the electrohydraulic theory has been able to give a reasonable understanding of the formalism of the observed phenomena in terms of well defined quantities. In this paper we have confined the discussions entirely to a theory based on physical state variables. This analysis does not preclude the possibility, that ionic events, for instance in terms of potassium and sodium-exchange, may be included in the above formalism, or may offer alternative explanations to the findings. The basic assumption of the presence of fixed charges (*i. e.* immobile ionic groups) in the submicroscopic architecture of the excitable tissue structures offers many possibilities for understanding the mechanism of ion and water transport. It is hoped that further work will elucidate possible relations between the physical events and these membrane transport phenomena.

The costs of the equipment used in this work have been defrayed by grants from The Swedish Medical Research Council, The Swedish Cancer Association and the U. S. Air Research and Development Command (Grant No. AF 61 (052)—363), which are gratefully acknowledged. — My thanks are also due to Miss EBON HELLSTRÖM for valuable assistance throughout the work.

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The Range of Effect of the Sympathetic Vasodilator Fibres with Regard to Consecutive Sections of the Muscle Vessels

By

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Received 4 April 1961

Abstract

Folkow, B., S. Mellander and B. Öberg. *The range of effect of the sympathtic vasodilator fibres with regard to consecutive sections of the muscle vessels.* Acta physiol. scand. 1961. *53.* 7—22. — The effect of the activation of the sympathetic cholinergic vasodilator fibres has been studied by a technique permitting an analysis of the reactions in the pre- and postcapillary resistance vessels and the capacitance vessels of a skeletal muscle region. The experiments indicate that the dilator fibres are distributed essentially to the bigger precapillary resistance vessels of the muscles, where they can induce almost maximal dilatation, while it is likely that other consecutive vascular sections more or less completely lack such an innervation. The data do not support the view that the dilator fibres open up non-nutritional specific 'shunt vessels', but the above-mentioned fibre distribution may in certain experimental situations create a 'functional shunting' by causing an uneven distribution of capillary flow. A central excitation of these fibres in alarm situations, such as has been suggested, would thus mean an immediate massive blood flow increase in the skeletal muscles, with little or no regional blood 'pooling' or opening up of closed capillaries. However, as soon as the skeletal muscles are brought into action, the local production of vasodilator metabolites will open up precapillary sphincters, allowing the already established big flow to be distributed over the entire capillary network.

Though the distribution and the functional significance of the sympathetic cholinergic vasodilator nerves have been much studied during recent years, there are still many details which are poorly understood. The fact that they are centrally controlled from the cortex and hypothalamus, but not engaged in reflex blood pressure control, further that they are only distributed to the vessels of the skeletal muscles, and, possibly, also to the coronary vessels, in itself suggests a functional engagement, under circumstances where centrally induced, prompt increases of blood supply to the muscles are needed. However, the functional significance of these centrally induced reaction patterns is so far not definitely clear, though recent data make it highly probable that the sympathetic dilator fibres play an important role in the so-called defense reaction (ABRAHAMS, HILTON and ZBROZYNA 1960). — On the other hand, recent experiments (HYMAN *et al.* 1959, ROSELL and UVNÄS 1960) have been interpreted as indicating that the sympathetic vasodilator fibres should open up non-nutritional vessels or 'shunts', so that the increased flow should in fact bypass the muscle capillaries and hence not serve the metabolism of the muscles directly. This interpretation led to the suggestion (UVNÄS 1960) that the dilator fibres might be activated in circumstances where the animal rather has to conserve its oxygen resources. If so, their functional significance should be the very opposite of their proposed engagement in the defense reaction, insofar as in the latter case it is implied that the dilator fibres are activated with the ultimate purpose of increasing the nutritional blood supply of the muscles.

As in this department a technique has been developed (MELLANDER 1960) which under certain circumstances allows a quantitative and selective recording of neurogenic and hormonal effects on both the pre- and postcapillary resistance vessels and the capacitance vessels, it was thought of interest to study whether the sympathetic vasodilator fibres are distributed to all the mentioned vascular sections or only to some of them, and then also to which extent this section, or sections, can be influenced by the dilator fibres. Such data might shed further light on the problem of the functional significance of the sympathetic vasodilator fibres. The present results have previously been briefly outlined (FOLKOW 1959).

Method

The experiments have been performed on cats anesthetized with chloralose and urethane in amounts not exceeding 50 mg and 100 mg per kg of bodyweight, respectively. — With regard to measurement of changes in blood flow and blood volume, a method recently described in a paper by MELLANDER (1960) was used. This paper should be consulted for details concerning technique and analysis of the recordings. In principle, the hind parts of the cat were completely isolated from the upper parts of the animal at the level of the hips, with exception for the aorta, the inferior caval vein and the abdominal sympathetic nerve trunks, containing the great majority

of the vasomotor fibres running to the isolated hind parts. After removal of the intestine, the inferior mesenteric artery was cannulated to measure the arterial 'inflow pressure' of the hind parts. — The hind parts were enclosed in a water-filled temperature-regulated plethysmograph, by way of which the phasic shifts in tissue volume, and hence blood volume, could be continuously and quantitatively recorded. As in these experiments the interest was concentrated on blood flow in the skeletal muscles, the tail and the hind paws were excluded from the circulation by tight ligatures at the ankles and the proximal end of the tail. This left the blood supply intact to a skin volume comprising about 5—8 % of the total enclosed tissue volume. In a few experiments the cutaneous circulation was almost entirely excluded by skinning the hind limbs, with exception of a narrow skin strip surrounding the tissues at the hip level, which was used to seal the plethysmograph. The plethysmograph was in these cases filled with Tyrode solution at a temperature of 35° C, instead of water. — In order to measure the blood flow of the hind parts, a modified Gaddum recorder was inserted in the inferior caval vein, which in this type of preparation forms the sole outflow pathway. Further, the height at which the Gaddum recorder was mounted above the animal set the venous outflow pressure level, and this level could easily be adjusted by raising or lowering the recorder. Also, the arterial inflow pressure of the hind parts could, if desired, be kept constant during an experimental procedure, by adjustment of a screw clamp, applied around the aorta proximally to the site of pressure recording in the inferior mesenteric artery. — In this way phasic changes of blood flow, blood volume, and, in certain circumstances, also of net transcapillary fluid exchange, induced, for example, by vasomotor nerve activations or injections of vasoactive drugs, could be continuously recorded, while at the same time the pressure drop across the vascular bed could be kept essentially constant. In order to study the influence of the sympathetic cholinergic dilator fibres, as compared with that of intra-arterially applied acetylcholine on the different vascular sections of the hind parts, two different procedures were used.

1. In the first series the abdominal sympathetic trunks were centrally cut and cautiously isolated in order to allow direct stimulations of their peripheral ends approximately at the height of the border between the fourth and fifth lumbar vertebra. In ten of these experiments the sympathetic vasoconstrictor fibres were acutely blocked by dihydroergotamine, given as an intra-arterial infusion into the hind parts. In five experiments the animals had been chronically depleted of catechol amines by treating them with reserpine (Serpasil ®), about 2 mg/kg, for 4 days before the actual experiment. Thus, direct stimulations of the abdominal sympathetic trunks in this series of experiments caused an activation of the sympathetic dilator fibres, the vascular effect of which was fairly undisturbed by any concomitant excitation of the adrenergic vasoconstrictor fibres. The stimuli were delivered by a Grass stimulator at frequencies varying between 1—20/sec, a voltage of 4—8 and a pulse duration of 5 msec. — Cloise arterial injections or infusions of acetylcholine and other drugs could be performed *via* the cannulated central stump of one of the lumbar arteries.

2. In the second series (10 exp.) attempts were made to activate the sympathetic dilator fibres from their diencephalic integration centre in the anterior parts of the hypothalamus. For this purpose the head of the animal was mounted in a Horsley-Clarke apparatus, allowing a fairly exact localization of bilateral, concentric stimulation electrodes. Square-wave stimuli at a voltage of 2—5, a pulse duration of 2—5 msec and a frequency between 30—90/sec, were delivered from a stimulator, Grass model S 4 C. In the course of these experiments the sympathetic dilator fibres of the hind parts were blocked by atropine, and in a few experiments an essentially regional block of the constrictor fibres was induced by a slow intra-arterial infusion of dihydroergotamine into the hind parts.

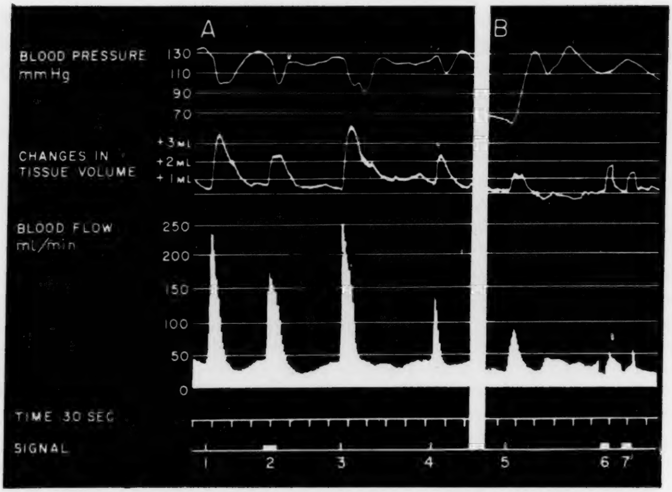

Fig. 1. Serpasile-treated cat. 3.5 kg. Chloralose-urethane.
A. Vascular response in the hind quarters of:
i. a. injection of acetylcholine (0.5 μg) (1);
stimulation of lumbar symp. vasodilator nerves (20 imp/sec). (2);
i. a. injection of acetylcholine (2 μg) (3)
i. a. injection of acetylcholine (0.01 μg) (4)
B. Effects on blood flow and 'blood volume' in the hind quarters induced by
rise of arterial pressure 70 mm Hg (5)
rise of venous outflow pressure 6 mm Hg (6)
rise of venous pressure 3 mm Hg (7); (for further description see text).

Results

1. *Effects of stimulation of the abdominal sympathetic trunks.* In these experiments the vasoconstrictor fibres had, as has been mentioned, either been blocked by dihydroergotamine in the course of the experiment, or by reserpine, given to the animals during the preceding four days. Both types of experiments gave essentially similar results, but the constrictor fibre block was throughout more complete after reserpine treatment. This was made obvious by the fact that even maximal sympathetic excitation did not induce any vasoconstriction whatsoever in most of these experiments, when the dilator fibres had been blocked by atropine. After dihydroergotamine, on the other hand, a somewhat delayed, moderate vasoconstrictor response was revealed under such circumstances, indicating that the vasoconstrictor fibres were not completely blocked by this drug. For such reasons the experiments on the reserpine-treated animals were in some respects best suited for a detailed analysis of the distribution of the dilator fibres to the different consecutive vascular sections. Further, they provided the most clear-cut data concerning the extent of the dilator fibre

effect on the muscle blood vessels, as compared to the maximal vasodilator response which could be obtained by intra-arterial injections of huge doses of vasodilator agents. Acetylcholine was used here, because, under other circumstances, we have observed that the vasodilatation induced by massive muscular work is of the same magnitude as that obtained by big doses of acetylcholine and, furthermore, such a maximal exercise hyperaemia cannot be further increased by addition of acetylcholine or other blood-borne vasodilator agents. Such observations indicate that acetylcholine can be used for inducing a complete inhibition of all those vascular smooth muscles, whose tone adds to the flow resistance.

Fig. 1 illustrates part of a typical experiment which from a technical point of view was entirely satisfactory and performed on a reserpine-treated animal. Generally, the arterial blood pressure in such animals is quite low, which to a great extent appears to be due to the fact that the nervous adrenergic control of the venous side and the heart is eliminated by the drug. However, if only the filling pressure of the heart was somewhat raised, which could easily be done by adjusting the height of the inflow from the funnel of the flow recorder, it was possible to maintain a fairly 'normal' blood pressure, in spite of the pharmacological adrenergic 'sympathectomy'. As also the vagal nerves had been cut in the neck region, reflex blood pressure control was thus eliminated, and therefore the inflow rate from the funnel to the heart had to be very exactly balanced, if the blood pressure was to be kept fairly constant. Even minor shifts of the filling pressure of the heart could induce dramatic pressure changes, presumably by changing stroke volume and hence cardiac output, a circumstance which was utilized when the experimental procedure required sudden changes in the arterial inflow pressure. — The 'resting' blood flow was of the order of 6—8 ml/min/100 ml of tissue, and could be increased about five times by supramaximal amounts of intra-arterially injected acetylcholine, *i. e.* to about 35 ml/min/100 ml of tissue. Thus, if the blood pressure was kept within the normal range, 'basal vascular tone', — the extent of tone left after elimination of the vasoconstrictor fibres — was essentially normal within the skeletal muscles of the reserpine-treated animals, in spite of the fact that they were completely depleted of catechol amines. It was further observed that the resistance vessels exhibited an often very striking 'autoregulation', which incidently is clear from the relation between pressure and flow in '5' of Fig. 1. B., a finding which will be dealt with in more detail in another publication (Folkow and Öberg 1961). — The biggest increases of blood flow, obtained by maximal excitation of the sympathetic vasodilator fibres at the highest physiological rates, 10—20 impulses/sec, were slightly above 25 ml/min/100 ml of tissue; the sympathetic stimulation illustrated in Fig. 1 A, '2', resulted in a peak flow of about 25 ml/min/100 ml of tissue, but in the preceding stimulations in the same experiment somewhat higher flow values were reached.

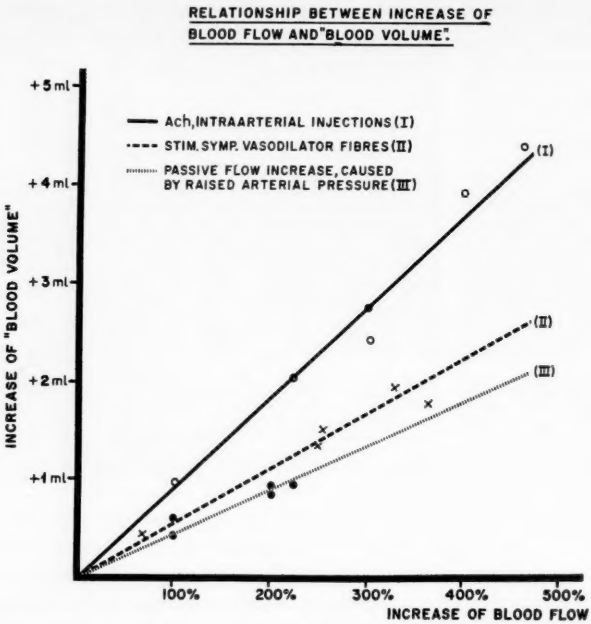

Fig. 2. Relationship between increase of blood flow and blood volume in the hind quarters of a 3 kg cat as induced by i. a. injections of acetylcholine (I), stimulations of the lumbar symp. vasodilator fibres (II) and by rises of arterial perfusion pressure (III). The data are obtained from the experiment part of which is shown in Fig. 1.

The maximal flow increases that could be induced by the vasodilator nerves were thus only about 20—25 % smaller than those obtained by supramaximal amounts of acetylcholine. It should firstly be realized, that the studied hind parts also contain some skin and bone tissue, roughly 5 and 10 volume per cent, respecively, and the blood flow of these tissues, was, of course, included in the flow measurement. The vasodilator fibres are known to be distributed only to the skeletal muscles but the intra-arterially injected acetylcholine will reach all the tissues included in the studied hind parts. Secondly, for technical reasons it can hardly be expected that sympathetic stimulation at the level of the fourth lumbar vertebral disc will activate all the vasodilator fibres of the skeletal muscles, the blood flow and volume of which are included in the measurement. Those dilator fibres which are distributed to the vessels of skeletal muscles around the hip level, will presumably have left the sympathetic trunk at a more cranial level, as the innervation of some of these muscles originates from more cranial parts of the spinal medulla. Furthermore, some of these fibres run the risk of being damaged when the animal is 'divided'

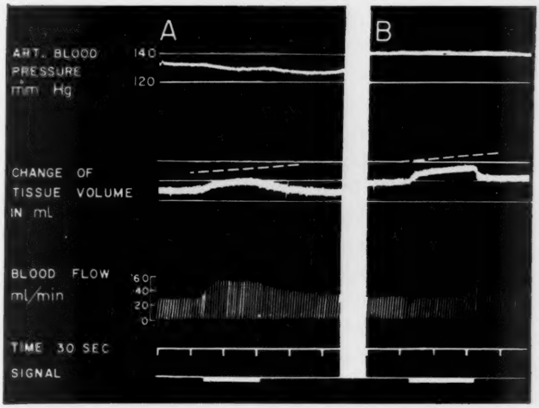

Fig. 3. Serpasile-treated cat, 3.2 kg. Chloralose-urethane. *A*. Stimulation of sympathetic vasodilator fibres at successively increasing frequencies (3—6 imp/sec) so as to induce a sustained vasodilatation. Note that after the slight initial increase of tissue volume, there is a slower and continuous increment (indicated by adjacent dashed line) suggesting a phase of outward filtration of fluid. *B*. Typical 'filtration slope' in the volume curve induced by raising venous outflow pressure 3 mm Hg.

by mass ligatures at the hip level. Thirdly, any damage inflicted on the abdominal sympathetic trunks when prepared for stimulation, will, of course, tend to diminish the extent of the vasodilatation obtained on their stimulation. Thus, a number of factors, impossible to avoid completely in experiments of this type, will tend to diminish the extent of blood flow increase caused by maximal dilator fibre excitation, as compared to that obtained by supramaximal amounts of blood-borne vasodilator agents. Such circumstances make it probable that the blood flow increase in the skeletal muscles, which can be induced by intense sympathetic vasodilator fibre activation, is, — if anything, — even closer to the maximally obtainable muscle blood flow values in the intact animal than would be expected from the actual measurements in Fig. 1 and the diagram of Fig. 2. These questions will be further discussed below when the functional significance of these nerve fibres will be briefly considered. — Incidently, it was a striking and constant phenomenon that, whatever frequency and stimulation characteristics were used to activate the vasodilator fibres, it was not possible to maintain an increased blood flow for more than half a minute or so, if the stimulation frequency was not continuously raised throughout the stimulation period. It was, in fact, necessary to increase successively the stimulation frequency in the experiment illustrated in Fig. 3, as the event to be studied here made it necessary to try to maintain the flow increase at a steady level for some period of time. Even in such circumstances, however, a steadily increased flow could never be maintained for more than about a minute, which is in striking contrast to the longlasting, fairly steady vascular effect that can be induced by a continuous constrictor fibre stimulation. The background of this difference between the two types of sympathetic vasomotor fibres is obscure; it may be that the vasodilator fibres are more easily damaged by the artificial stimulation, though this can hardly

be the sole explanation. Another possibility is that, in some way or other, they may be functionally adapted mainly for inducing phasic decreases of vascular tone, while the constrictor fibres must, of course, be fitted for their tonic vascular control. Whatever the case, this phenomenon appears to make it almost impossible to analyse such events in the neurogenically induced dilatation that require a steady state over any length of time.

It should also be observed in Fig. 1 that the increase in tissue volume appears to be relatively bigger for a given flow increase when acetylcholine is injected than when the dilator fibres are activated (*cf.* for example, '2' and '4'). In the dihydroergotamine-treated animals, where constrictor fibre block is not complete, it was often observed that a flow increase was combined with almost no increase in volume on sympathetic stimulation; sometimes even a decrease on volume was obtained. Acetylcholine always increased blood flow and tissue volume markedly.

The diagram of Fig. 2 is based on all the effects recorded in the experiment, part of which is illustrated in Fig. 1, and shows the relationship between flow increase and volume increase, as caused by intra-arterially injected acetylcholine (curve I), by sympathetic vasodilator fibre activation (curve II) and simply by raising the arterial pressure level (curve III). It can be seen from this diagram, which is representative of all the technically successful experiments, that — for each level of blood flow increase — the increase of regional blood volume appears to be almost twice as big when acetylcholine is distributed *via* the blood stream, as when the same agent is locally liberated as the transmitter at the cholinergic vasodilator nerve endings. No doubt, however, the dilator fibres also cause some increase in regional blood volume if they are selectively activated, but it must be remembered that this is inevitable, even if the fibre distribution should be essentially restricted, say, to the precapillary resistance vessels, and this for two reasons. From earlier studies it is known that the hind parts of a medium-sized cat contain roughly some 20 ml of blood (Mellander 1960). It can be approximately calculated that some 3—5 % of this blood is contained within the true arterioles (see Green 1950, Table 1), which means that these vessels of the hind parts at 'normal' vascular tone contain at most 1 ml of blood. If a selective widening of only these vessels took place, big enough to increase blood flow four times, this would — according to Poiseuille's law — imply a 40 % increase in internal radius and a doubling of their blood content, in the present case raising the total blood volume of the hind parts by roughly 1 ml. Such an approximate deduction makes it clear that in a way the smallest arteries also form a part of the functionally defined 'capacitance vessels', the most important section of which is constituted by the veins. — Furthermore, even if the dilator fibres cause a relaxation only of the precapillary part of the resistance vessels, this dilatation must so change the profile of the pressure drop curve along the vascular tree as to raise the distending pressure in the capillaries and the

distensible veins. Thus, other factors being constant, any dilatation of the precapillary resistance vessels must increase the venous blood content, by way of passive-elastic distension, even if their smooth muscle tone is completely unaffected by the sympathetic vasodilator fibres. — Curve III in the diagram of Fig. 2 illustrates the effect on blood flow and regional blood volume as induced simply by increasing the arterial pressure without and primary change in vascular smooth muscle tone. It can be seen from this curve that the passive-elastic volume increase for a given increase in blood flow is very nearly the same as that caused by sympathetic vasodilator fibre stimulation. This finding is also illustrated by '5' in Fig. 1 B. '6' and '7' in Fig. 1 B illustrate in another way how even slight increases in mean pressure within the venous section will definitely affect its contained blood volume. On the basis of haemodynamic considerations it can be deduced that — for a given increase of flow — mean venous pressure should be increased to roughly the same extent, whether the flow increase is due to arteriolar dilatation or to raised arterial pressure. Provided that no changes of venous tone take place, venous distension can therefore be expected to be fairly similar in the two cases. The slight difference between curves II and III, is, in fact, to be expected because — as mentioned above — the relaxation of the resistance vessels upon dilator fibre activation means an additional, small blood volume increase besides that due to venous distension. On the other hand, if venous smooth muscle tone had also been inhibited by vasodilator fibre activation, the volume increase could be expected to equal that caused by acetylcholine injections, where presumably all vascular sections are reached by the dilator agent. Thus these findings strongly suggest that the most important section of the capacitance vessels — the veins — is supplied with only few, if any, vasodilator fibres. It should then be remembered that the veins at the same time constitute the postcapillary section of the resistance vessels. — It can also be seen from Fig. 1 and 2 that the volume increases in absolute values are rather small, compared with the contained blood volume in the hind parts. The reason for this is that venous tone, when deprived of its constrictor fibre control, is fairly low, compared with the obviously rather marked 'basal tone' of the resistance vessels. The extent of volume increase caused by acetylcholine injection was far bigger if the initial tone of the capacitance vessels had been raised by an intra-arterial infusion of, for example, noradrenaline.

Fig. 3 illustrates in another way the fact that vasodilator fibre activation evidently causes an increase of intravascular pressure at and beyond the capillary level, which should also be the case if these fibres are predominantly or solely distributed to the precapillary section of the resistance vessels. In this experiment a moderate, but steadily maintained blood flow increase was induced by dilator fibre stimulation, which, as mentioned earlier, made it necessary to increase the stimulation frequency continuously. The onset of stimulation is accompanied by an initial, rather rapid phase of flow and volume

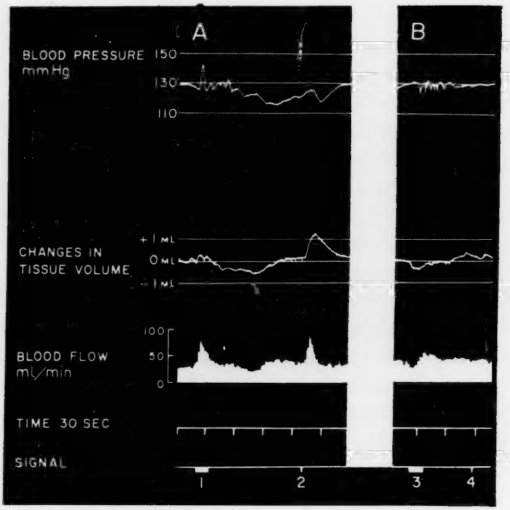

Fig. 4. Cat 3.2 kg. Chloralose-urethane.
A. Vascular responses in skeletal muscles of the hind quarters of the cat, evoked by topical hypothalamic stimulation activating the cholinergic vasodilator nerves (1), and by close arterial injection of 0.01 μg acetylcholine (2). Although blood flow is about equally increased in both cases, the regional tissue volume (blood volume) is increased by acetylcholine but hardly affected during hypothalamic stimulation.
B. The same experimental steps as in *A*. performed after atropinization. The vasodilator responses are now abolished. A slight vasoconstriction is obtained by hypothalamic stimulation (3).

increase, in time well co-ordinated with each other. Once blood flow is stabilized at its higher level, however, it is accompanied by a slow but continuous increase of tissue volume (A in Fig. 3), which in character is very similar to that induced by a slight rise in venous pressure (B in Fig. 3). In the analysis of the experimental technique used, Mellander (1960) presented strong evidence to show that such a secondary, continuous volume increase is due to an outward filtration, caused by raised capillary pressure. As here arterial inflow pressure and venous outflow pressure are kept constant, this effect of dilator fibre stimulation suggests that the main resistance decrease is located proximally to the capillary level. Owing to the consequent shift in profile of the pressure drop along the vascular bed, it can be expected that venous pressure must also be somewhat raised, leading to a passive venous distension. Always, when it proved technically possible to maintain a steady level of increased flow by dilator fibre stimulation long enough to perform such a measurement, there was evidence of outward filtration. If a steady flow level cannot be maintained, the present technique does not allow an exact evaluation of the fraction of the volume shift that is due to transcapillary filtration exchange. It can be expected, however, that the extent of outward filtration is much more marked at, for instance, maximal activation of the dilator fibres, but as soon as huge flow increases are induced, they are, as mentioned, always very transient.

2. *Effects of hypothalamic stimulation.* In these experiments the sympathetic vasodilator fibres were activated by topical stimulation of their diencephalic integration centre. The effect of a hypothalamic stimulation on regional muscle

blood flow and blood volume is illustrated in Fig. 4, '1', where the activation of the dilator fibres causes a blood flow increase of about 150 %. To eliminate the passive changes of blood flow and blood volume, due to blood pressure changes in connection with the stimulation, the arterial inflow pressure was kept fairly constant by adjusting a screw clamp placed around the abdominal aorta. — It can be seen from the figure that the prompt blood flow increase is combined with very little shift in tissue volume, followed by a somewhat delayed phase of volume decrease. On the other hand, injection of acetylcholine (Fig. 4, '2'), in a concentration that gives about the same blood flow increase, causes a clear-cut increase also in volume. Atropine eliminates the acetylcholine effect and the increase of flow on hypothalamic stimulation, leaving a slight decrease in flow and volume as the only regional vascular effects consequent to the stimulation. — The effect of hypothalamic stimulation on the tissue volume varied to some extent; in some experiments there was an actual decrease in volume at the peak of the flow increase, in others the volume increased slightly, but always to a lesser extent as compared with the effect of injected acetylcholine in amounts that elicited an equal blood flow increase. — It was mentioned in the previous section that the dilator fibres appear to be distributed essentially to the precapillary resistance vessels, but that their activation nevertheless must lead to a certain volume increase, due to a passive distension of the venous side. It does therefore, in fact, appear that the topical hypothalamic stimulation, — beside the activation of the vasodilator fibres —, also to some extent activates vasoconstrictor fibres to the capacitance vessels of the muscles. Otherwise it can hardly be explained how sometimes blood flow was increased by hypothalamic stimulation, while there was no change or even an actual decrease in tissue volume.

Thus, the results of the hypothalamic excitation of the sympathetic vasodilator fibres also suggest that they are essentially distributed only to the resistance section of the vascular bed in skeletal muscles. Furthermore, it appears that the cardiovascular reaction pattern, which is induced from their diencephalic centre, may to some extent also involve activation of the vasoconstrictor fibres of the capacitance section of the vascular bed in the muscles.

Discussion

The present experiments indicate that, under favourable experimental conditions, the sympathetic vasodilator fibres, when activated at the highest physiological rates, are capable of inducing blood flow increases in the skeletal muscles which in extent are close to the state of maximal dilatation. As, further, a number of unavoidable experimental artifacts, — unlikely to affect the estimation of maximal vasodilatation —, tend to diminish the neurogenic vasodilator response, it is in fact probable that the present results, if anything, underestimate the potency of the vasodilator fibres.

The question then arises whether the sympathetic vasodilator fibres really affect the same set of vessels within the muscles as the metabolites, which are locally released in muscular work. As briefly mentioned in the introduction, a series of studies of the dilator fibre effect on the relationship between volume flow and diffusion exchange have led to the suggestion that the dilator fibres might open up non-nutritional 'shunts', parallel-coupled to the capillary bed. No doubt stimulation of these fibres can in some experimental circumstances create a situation that deserves to be called 'functional shunting'. For the evaluation of the phsyiological significance of the dilator fibres it is, however, most important to distinguish between true shunt vessels and the occurrence of a functional shunting due, for example, to an uneven distribution of the capillary flow. For such reasons this problem will here be dealt with in some detail, evaluating the present results against the background of a number of well-established data on muscle blood flow and oxygen consumption.

If, for a moment, it is presumed that true shunt vessels, controlled by the cholinergic vasodilator fibres, exist, acetylcholine can then hardly be imagined to lack the power of relaxing these shunt vessels when distributed *via* the blood stream in high concentrations. The injected acetylcholine must, by way of the capillary walls, promptly reach the tissue fluid which form the immediate environment of all cells, including the presumed shunts and their cholinergically innervated smooth muscles. To draw a parallel, the adrenergically innervated shunts in the skin certainly respond sensitively to catechol amines in the blood. For such reasons it has to be assumed that the maximal flow value, obtained by supramaximal amounts of intra-arterially injected acetylcholine must also include the blood flow increase through the shunts, if they really exist. — Further, acetylcholine and related substances are known to dilate the 'nutritional' blood vessels of the muscles powerfully, being in fact able to induce the same extent of maximal flow as can be elicited by intense muscle activity. Acetylcholine, intra-arterially injected at the peak of a maximal work hyperaemia, is in our experience not able further to enhance the blood flow increase. Now, if it is tentatively assumed that specific muscle shunts exist, such findings must mean either that both the 'nutritional' vessels and the shunts are dilated by the locally released metabolites, or that the shunts are unaffected both by the metabolites and by injected acetylcholine. As mentioned above, the latter alternative is highly unlikely and therefore it is necessary to consider the possibility that muscular work also causes a dilatation of the presumed shunts.

When this question is dealt with, it must be remembered that the vasodilator fibres can induce almost the same extent of flow increase as supramaximal concentrations of acetylcholine or intense muscular work. To take some actual figures from the experiment, illustrated in Fig. 1 and 2, maximal dilatation here implied a blood flow of 35 ml/min/100 ml of tissue, while intense dilator fibre activation increased flow slightly above 25 ml/min/

100 ml of tissue. The 'resting' blood flow in the studied tissue part, consisting of up to about 85 % skeletal muscle, is in our experience around 3—4 ml/min/100 ml of tissue, under circumstances where a normal resting constrictor fibre tone is present, and about twice as high when the vessels are sympathectomized. If true shunt vessels really exist, it then follows that the margin left for increasing the nutritional blood flow of the muscles would be at best some 300 % in the intact animal, *i. e.* it would be constituted by the difference between the flow figures of 35 ml and 25 ml. As mentioned earlier, this difference may in reality be even smaller, as with the present technique the extent of the dilator fibre influence is — if anything — underestimated. One is thus forced to assume that the greater fraction of the increased cardiac output in muscular work in fact bypasses the muscle capillaries, if true shunts exist, being of no use for the nutrition of the muscles but only adding to the load on the heart.

Already the above-mentioned considerations thus make it clear that the existence of any significant extent of specific shunts within the skeletal muscles is highly unlikely. In addition, the above-mentioned flow figures, — if tentatively interpreted in terms of a dual shunt and 'nutritional' muscle circulation, — are incompatible with known data for the relation between maximal oxygen uptake in intensely working muscles and their maximal blood flow. If we here exemplify with data taken from man, a resting subject can be considered to consume about 250 ml oxygen/min, where roughly 20 % can be calculated to be consumed by the skeletal muscles, at a muscle blood flow of about 2—4 ml/min/100 ml of tissue. The blood flow can at best be increased some 15—20 times, to about 40—50 ml/min, while during heavy muscular work the oxygen extraction per unit blood volume can increase roughly 3 times. During intense muscle activity the total oxygen consumption of the muscles can increase well above 50 times, as the maximal oxygen uptake in man then often reaches 3,500—4,000 ml/min, most of which is taken up by the muscles. It is mathematically impossible to account for this huge increase of oyygen uptake by the working muscles, if any significant fraction of their maximal blood flow bypassed the capillaries. Further, the venous blood coming from intensely working muscles can be almost depleted of oxygen, which would not have been the case if a bigger fraction of it had passed shunts. Data concerning maximal oxygen uptake, etc., in cats are not available, but it is hardly likely that they would be profoundly different from those in man, when corrected for the difference in body size. Thus, there appears to be no room left for any specific shunts in the muscle circulation, if it is not assumed firstly that they are normally closed in a state of intense tonic contraction and only opened by activation of the dilator fibres, secondly that their cholinergically innervated smooth muscles are entirely unaffected even by massive concentrations of acetylcholine and vasodilator metabolites in their immediate environment. Such assumptions are so untenable that it seems necessary to reject the theory

that any significant number of specific shunts exist in the muscles. Other ways must then be found to interpret the series of data which undoubtedly suggest the establishment of a 'functional shunting' when the dilator fibres are experimentally activated (Hyman *et al.* 1959, Rosell and Uvnäs 1960).

The present study indicates that the vasodilator fibres are mainly distributed to the precapillary resistance vessels, because, — when related to the same flow increase induced by injected acetylcholine, — their activation causes a much smaller increase of regional blood volume, not bigger than can be predicted to be the result of a selective arteriolar dilatation and the consequent passive-elastic distension of the venous side. It appears therefore that the venous side, constituting the postcapillary resistance vessels and also the main part of the capacitance vessels, is not directly influenced by the dilator fibres. Also, the fact that the dilator fibres appear to bring about a raised mean capillary pressure is in harmony with such a selectivity in fibre distribution. — Suppose now that the dilator fibres only make contact with more proximal sections of the precapillary resistance vessels, *i. e.* the true arterioles, but not with the smallest arterial ramifications, which can be called the 'precapillary sphincter sections', controlling the number of capillaries open to flow. A selective, intense activation of the dilator fibres will then bring about a markedly increased flow, — but not a maximal one, as not all vascular sections contributing to the flow resistance are widened. This increased flow will, however, pass essentially the same, limited fraction of the capillary bed that is normally open in the resting muscle. It can even be expected that some of the capillaries may become closed, as in electrical stimulations it is unavoidable to induce some vasoconstrictor fibre effects also, except in animals severely treated with reserpine, and the constrictor fibres also affect the precapillary sphincter sections to some extent (see *e. g.* Folkow 1955, Folkow and Mellander 1960 and Rosell and Uvnäs 1960). — Furthermore, it is well-known that the smooth muscles of the vascular sphincter regions relax and open up previously closed capillaries when tissue metabolism is increased. They may react in a similar way when, for instance, blood flow is artificially reduced, other things being constant. Hence it is not unlikely that they react in the opposite direction, *i. e.* constrict somewhat, when an increased blood supply is suddenly forced upon them by an upstream, strictly localized dilatation, because of the consequent changes in their immediate chemical environment and the regional increase in transmural pressure (Folkow and Öberg 1961).

Thus, an experimental situation may here be created, where an increased volume flow passes through an unchanged, or even somewhat rarified, capillary network. As has been previously briefly discussed (Folkow 1960), such a situation implies firstly a reduced time for diffusion exchange in the individual capillary, secondly an unchanged or even a somewhat reduced capillary surface available for this exchange, and thirdly a slightly increased mean diffusion distance from the blood to the tissue cells. This means that limited

sections of a skeletal muscle are provided with a great excess of nutritional supply, while other parts of the muscle get little or nothing. In other words, a 'functional shunting' is experimentally created, where no specific shunts or any new concepts of vascular smooth muscle behaviour are implied, only a distribution of the dilator fibres restricted to the bigger precapillary resistance vessels. Such a view is in harmony with recent studies on capillary permeability in skeletal muscles (Renkin 1959, a, b), which suggest that 'shunting' of blood in skeletal muscles is merely a matter of a variable distribution of flow within the skeletal muscles, while no evidence of specific shunt vessels could be found. It appears in fact that available data on the characteristics of the dilator fibre effects, also those of Hyman *et al.* (1959) and Rosell and Uvnäs (1960), do not necessitate the implication of true shunt vessels, but can be explained along the above-mentioned hypothesis of a selective dilator fibre distribution. In quite recent experiments, utilizing a different experimental approach, Renkin and Rosell (1961) have arrived at a similar view. It is haemodynamically important to distinguish such a point of view, as there is a great difference between a vascular bed containing both nutritional vessels and parallel-coupled shunts and one with only the former type of vessels, where occasionally a type of 'functional shunting' can occur.

If this is so, the centrally induced visceromotor pattern, of which the dilator fibre activation forms a dominant part (see Uvnäs 1960), is excellently fitted for participation in alarm reactions, as Abrahams, Hilton and Zbrozyna (1960) suggested. The immediate, dramatic redistribution of an increased cardiac output, favouring the skeletal muscles, implies an anticipation of the nutritional demands, arising at the moment the skeletal muscles are brought into action in a flight or defence reaction. The main pattern of blood flow is then already centrally induced, and as soon as the muscles are activated, the locally released vasodilator metabolites only have to open up all the capillaries, to spread out the huge blood flow over the entire capillary surface of the muscles.

The present hypothesis of a restricted vasodilator fibre distribution to the bigger precapillary resistance vessels only, further implies that the increased flow can be established with a minimum of blood 'pooling' within the muscles, as few, if any, dilator fibres appear to run to the venous side. It may even be so that the blood volume contained in the muscles can decrease in situations when the dilator fibres are centrally activated in the intact organism. This would be possible if the constrictor fibres to the venous side of the muscle vascular bed were concomitantly activated to some extent, as appeared to be the case at least in some of the present topical stimulations of the hypothalamus.

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Physiological Adjustments to Prolonged Diving in the American Alligator

Alligator mississippiensis

By

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Abstract

ANDERSEN, H. T. *Physiological adjustments to prolonged diving in the American alligator, Alligator mississippiensis.* Acta physiol. scand. 1961. *53.* 23—45. — Conspicuous physiological adjustments to experimental submersion have been observed in the natural divers among the birds and the mammals. In order to obtain further information on the phenomena associated with water immersion, and to explore these characteristics in other diving vertebrates, experiments have been carried out on the American alligator, *Alligator mississippiensis.* The heart rate was found to slow down during diving. The pressure induced in the large arteries by ventricular systole diminished only gradually during diastole, probably due to an increased peripheral resistance resulting from a marked vasoconstriction in certain vascular beds. This thesis was supported by the fact that lactic acid formed in the muscles during diving did not appear in the blood until after emersion, suggesting that the blood flow through the muscles is greatly reduced while the animals dive. The alligator may recover easily from a submergence lasting until the oxygen stores are almost completely depleted. These results indicate that the "diving reflexes" occur in a wide variety of vertebrate divers belonging to different phylogenetic groups. They also support the following conclusions:

1) The circulatory adjustments, by shutting off the perfusion to certain vascular beds, enable the alligator to make the limited oxygen stores last throughout prolonged periods of submersion.

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2) The animal remains in a state of useful consciousness until the oxygen stores are almost exhausted. This serves to extend the safety margin for prolonged diving and ensure a complete utilization of the oxygen available.

Certain animals among the vertebrates exhibit a high degree of adaptation to aquatic life, although they are equipped with essentially the same respiratory and circulatory organs as terrestrial forms. Some of these animals are known to be able to remain under water for an extended period of time, and endure a very high degree of asphyxiation. While submerged, they may be observed at rest, as well as carrying out various activities, such as feeding, cruising or catching prey.

The physiological adjustments which permit the diving mammals and birds to endure prolonged periods of submersion have been studied by several investigators. Since BERT (1870) started these studies, important contributions have been made by RICHET (1899), LOMBROSO (1913), IRVING, *et al.* (1941 a, 1942), SCHOLANDER (1940), and SCHOLANDER *et al.* (1942). The subject was reviewed by IRVING (1939).

The observations of these investigators have indicated certain factors to be particularly important for prolonged diving:

1) Large oxygen depots, and selective and complete use of these stores.
2) Relative insensitivity to effects of asphyxiation on respiration.
3) Reduced metabolism during diving.

A fair amount of knowledge of the physiological adjustments to prolonged diving in birds and mammals has been established, whereas similar information on the poikilothermic divers is scarce. There are diving animals both among the amphibians and the reptiles which presumably would be well suited for experimental work. The amphibians are known to remain under water for extremely long periods of time, but in these animals the cutaneous respiration is extraordinarily large, and it may well be that the cutaneous component of respiration is adequate to maintain life during diving. The reptiles are, however, almost entirely dependent on their lungs for respiratory gas exchange. It is, thus, very desirable to study the diving reptiles in order to make our knowledge of the physiological function in the natural divers more complete from a comparative point of view.

Among the reptiles, the crocodilians are well suited for such a study. The crocodilians are semiaquatic. They never depart far from water, and when disturbed, they will try to escape into the water and submerge themselves. Their fitness in an aquatic habitat is also evident from their hunting. They catch their prey in water, or they pull the victim into the water and submerge him until drowning.

A few publications deal with the diving ability of the reptiles, but only to a very limited extent (DILL and EDWARDS 1931, JOHANSEN 1959, WILBUR 1960). The present investigation was carried out on the American alligator,

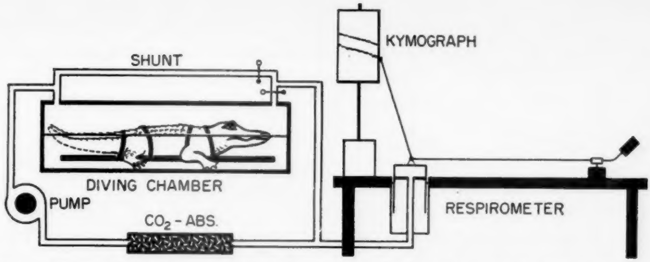

Fig. 1. Experimental design for the study of metabolism by indirect calorimetry.

Alligator mississippiensis, which, like the other crocodilians, is amphibious, and displays a striking ability to endure prolonged diving.

Materials and Methods

Animals and animal care

Fourteen alligators were used in the present study which was carried out from July 1958 to September 1959. The animals were kept in the laboratory at temperatures ranging from 22 to 27° C. They were fed horsemeat and bone meal. At times some of them had to be fed forcibly. None of the animals stayed at the institute for more than 3 months at a time. The weight of the animals varied little over the period which they spent at the institute.

The alligator is known to show marked seasonal changes as to activity and feeding habits. These changes were ignored in the present investigation because seasonal changes in the ability to perform prolonged diving did not occur as far as could be judged.

Arrangement of the diving experiments

Almost all of the data presented in this study has been obtained from experiments in which the animal was involuntarily submerged. The alligator was strapped down on a weighted board like the one shown in Fig. 1, so that diving could be induced by gently tilting the board. It was noticed that the animal would display the same characteristic adjustments to diving when only the nares were under water as they did when the body was completely submerged. Advantage was taken of this phenomenon in the experiments where the blood pressure recordings were done. Thermal disturbances during the experiments were avoided by insuring that the water in which the alligator was submerged was of the same temperature as the room in which the animals were kept.

Methods employed in the studies of the circulation

The heart rate was recorded between needle electrodes inserted subcutaneously into the right forelimb and the left hindlimb. Electrocardiograms were obtained both in the involuntary dives, and in dives performed spontaneously by an animal swimming freely and diving voluntarily. The recordings were obtained by means of a Sanborn Visette Electrocardiograph Model 300 or a Grass Electroencephalograph Model III D.

In order to secure information on the arterial blood pressure, the femoral artery was exposed, and a poly-ethylene catheter i. d. 0.6 mm was inserted approximately 5 cm into this vessel. The catheter was attached to a calibrated Statham pressure transducer Model P23AA. The pressures were recorded by a Gilson Electronics Mini-polygraph.

During the experiments dealing with measurements of blood pressures, only the nares of the animals were submerged. Diving conditions could therefore be accomplished by a minute tilting of the board, and changes in hydrostatic pressure due to this procedure could not be observed on the recording equipment. Sequences of the heart rate and the blood pressure were recorded continuously for at least 30 seconds at a time.

Methods used in the studies of respiration

Samples of arterial blood were withdrawn from the femoral artery which had been cannulated as described above. The blood samples were analyzed for their content of oxygen, carbon dioxide and lactic acid. The pH of the arterial blood was also measured. The syringes into which the blood samples were drawn contained heparin and a 10 % sodium fluoride solution in the dead space. A little mercury was drawn into the syringe after the blood in order to provide a gas tight seal, and to facilitate adequate mixing of the blood before an aliquot was removed for analysis. Until the analytical work could be performed, from 2 to 8 hours after sampling, the syringes were stored in a bath of ice and water. The technic for anaerobic handling of the blood samples outlined by ROUGHTON and SCHOLANDER (1943) was followed closely.

Since the oxygen of the blood constitutes a very important part of the total quantity of oxygen available during diving, the amount sampled for analytical purposes had to be kept at a minimum. This requires the exclusive use of microtechnics in the blood analysis. By means of such procedures, it was possible to keep the individual blood samples at 0.8 ml each as this amount proved sufficient for determinations in duplicate of all of the parameters referred to below, except for the pH which was always measured separately. To prevent working with anemic animals usually not more than two experiments involving sampling of blood were performed on any animal.

The content of oxygen in the blood was determined by means of the Scholander-Roughton Syringe Method (ROUGHTON and SCHOLANDER 1943). The reagents described in the original paper did not appear useful when applied to alligator blood. This finding was not surprising considering that the method was first developed for the blood of homoiothermic animals, and especially for mammalian blood. By developing a different set of reagents, SCHOLANDER and VAN DAM (1956) successfully analyzed blood from different species of fishes by the Syringe Method. This modified procedure also failed to work satisfactorily on alligator blood, however, because the solution No. 5, the acid sulfate solution, did not break down the blood proteins so as to constitute a flocculent precipitate in the syringe. By increasing the acidity of this solution so that it was made up of 30 g Na_2SO_4 anhydrous in 100 ml of water to which 8 ml concentrated H_2SO_4 was added, it was made applicable to alligator blood. The amount of carbon dioxide in the blood was also determined in the Scholander-Roughton Syringe as described by SCHOLANDER, FLEMISTER and IRVING (1947).

Determinations of the concentration of lactic acid in the blood samples were carried out according to the method of BARKER and SUMMERSON (1941) and modified by STRÖM (1949).

The pH of the arterial blood was measured by means of a Beckman Zeromatic pH-meter provided with a Beckman 39022 Hypodermic Style Electrode Assembly which permits pH measurements on 0.5 ml of blood while the sample is drawn.

The blood volume was estimated in two of the alligators by bleeding and washing out the vascular system with oxygenated Frog Ringer's solution. Such a wash-out method is especially well applicable to a poikilothermic, diving animal like the alligator because the heart will keep on beating for hours after almost all the blood has been drained, thereby ensuring an adequate wash-out. The oxygen capacity of the blood was determined on blood samples equilibrated with air and analyzed for oxygen as described above.

Gas from the lungs was sampled at suitable intervals during diving. For this purpose the trachea was cannulated with a piece of plastic tubing i. d. 0.8 mm which was inserted approximately 10 cm. A sampling bulb of capacity 6 ml was attached to the polyethylene tubing. Gas could be withdrawn from the lungs into the sampling bulb by lowering a mercury-filled levelling bulb connected to the sampling bulb. Before a sample was taken, the sampling bulb was filled with gas from the lung air, the latter being subsequently returned to the animal through the catheter. This procedure was repeated two to three times, thus, ensuring a certain degree of mixing of the gas in the lungs before the actual sample was removed. Not more than 2 ml of lung air was withdrawn in each sample. This amount of gas provided an ample supply for duplicate determinations of the content of carbon dioxide and oxygen in the sample as analyzed in the 0.5 ml Analyzer (SCHOLANDER 1947).

The lung volume of two of the animals was estimated by means of total body plethysmography. The plethysmograph was made from a heavy-walled plastic tube. A manometer tube was taken from a Van Slyke Manometric Blood Gas Apparatus and built into the plethysmograph by means of a plastic connector fitted with two o-rings which sealed around the manometer tube. The plethysmograph was also supplied with a small faucet into which the nozzle of a 20 ml syringe fitted very accurately. The animal was introduced into the plethysmograph which was subsequently filled with water and sealed off. The formation of air bubbles along the inside wall of the plethysmograph was avoided by coating with a very small amount of a detergent.

In an experiment an initial pressure P_1 was read off the manometer, the corresponding, unknown lung volume being V_1. A small amount of water ΔV, was introduced into the plethysmograph from the syringe. Hereby, the lung volume diminished to a value $V_2 = (V_1 - \Delta V)$. The pressure would simultaneously increase from P_1 to $P_2 = (P_1 + \Delta P)$ which could be read off the manometer. From this information V_1 is calculated as follows:

$$V_1P_1 = V_2P_2 = (V_1 - \Delta V)\,(P_1 + \Delta P)$$

$$V_1P_1 = V_1P_1 + V_1\Delta P - P_1\Delta V - \Delta V\Delta P$$

$$V_1\Delta P = P_1\Delta V + \Delta V\Delta P = \Delta V(P_1 + \Delta P)$$

$$V_1 = \frac{\Delta V(P_1 + \Delta P)}{\Delta P}$$

Indirect calorimetry for the study of metabolic rate

The consumption of oxygen before and after submersion was registered continuously on the kymograph of the closed circuit apparatus shown in Fig. 1. The diving chamber was ventilated by means of a pump. The carbon dioxide in the system was absorbed in a column containing Ascarite[1], and the gas lost in this way was replaced with 100 % oxygen from the respirometer. The diving chamber was made from plexiglas so that the animal could be observed during the experiment.

Results

Performance of prolonged diving in the alligator

Most of the alligators used for the present study were 2—6 years old. When they were free to swim and dive in a large pool, they were observed to submerge 30—60 min at a time.

[1] Ascarite (Registered Trade Mark), a sodium hydroxide-asbestos mixture with CO_2 absorbing properties.

Fig. 2. ECG-tracings taken before, during and after a 15 minutes dive, showing the development of the diving bradycardia.

Heart rate and electrocardiographic changes

A very conspicuous bradycardia was observed in the diving alligator. The heart rate decreased gradually rather than instantaneously, at least during the forced dives, and the period of time required to establish a constant, low frequency varied considerably in the different individuals. The changes in the electrocardiogram associated with diving are shown in Fig. 2 as recorded between right forelimb and left hindlimb. A transient arrhythmia was always observed to take place during the period in which the diving bradycardia developed, as a ventricular contraction might be followed by a prolonged diastole. In the earlier stages of the dive this prolonged diastole appeared quite irregularly, and its duration varied markedly. The corresponding electrocardiogram, therefore, consisted of groups of fairly regular heart beats separated from each other by an extended diastole. The ECG-tracings furnished in Fig. 2 show that the fully developed bradycardia is caused mainly by the prolonged diastoles. In the pre-diving period the total time required for the registration of the electrical events associated with one complete cardiac cycle was 1.30 sec.

The T-wave diminished rapidly at the onset of the dive, and during the

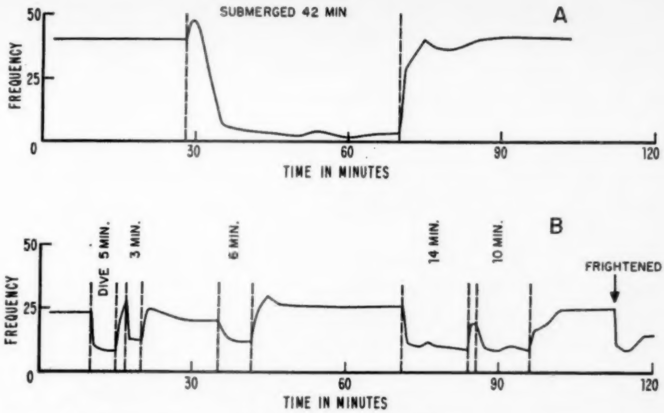

Fig. 3. A graphical representation of the diving bradycardia.
A. From an involuntary dive.
B. From a series of voluntary dives.

first minute of submersion it was hardly noticeable. When it reappeared, the amplitude showed a marked increase. The T-wave was frequently diphasic or completely inverted while the animal was submerged. The increased amplitude of this wave persisted usually for several minutes after emergence.

Fig. 3 A is a graphical representation of variations in the heart rate during an experiment in which the alligator endured involuntary submersion for 42 min. The heart frequency was 41 beats per minute in the pre-diving period. An initial, slight increase in the heart rate took place before the bradycardia started developing. Within 10 min the frequency of the heart had dropped to 2—3 beats per minute. The bradycardia persisted during periods of vigorous struggling in conformity with observations on the seal (Scholander 1940), and the duck (Andersen 1959 a). The data shown in Fig. 3 B was obtained from an animal swimming freely in a large tub, diving voluntarily. In this case, the pre-diving heart rate was 24 beats per minute. The frequency of the heart did not drop down to such an extremely low level as the one shown in Fig. 3 A, but was maintained at 30—50 % of the pre-diving rate. However, the time required for the establishment of this degree of bradycardia was much less than in the forcibly submerged animal. The pre-diving heart rate was rapidly restored upon emersion from involuntary dives as well as from voluntary ones.

In one experiment in which the right vagus was cut in one of the alligators, no bradycardia developed during diving.

Arterial blood pressure

Experiments were conducted in which the changes in arterial blood pressure during diving was studied in the femoral artery. Sections of a record obtained

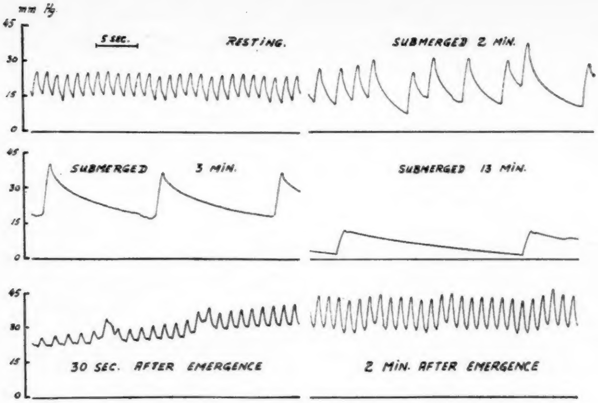

Fig. 4. Tracings showing the arterial blood pressure in the femoral artery during a 20 minute dive.

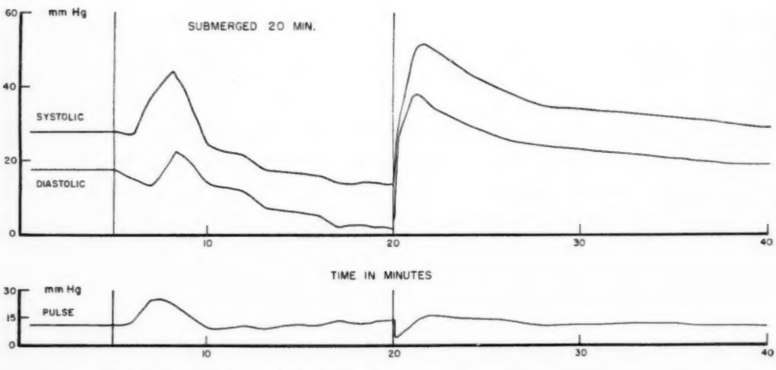

Fig. 5. A graphical plot of the systolic, diastolic and pulse pressures in the femoral artery of a diving alligator.

in one of the experiments are presented in Fig. 4. The systolic, diastolic and pulse pressures from this same experiment were plotted graphically from the continuous record and are given in Fig. 5. The average value for these three pressures was 28, 17 and 11 mm Hg respectively in the pre-diving period. During the earliest part of the dive, all three pressures increased very markedly. After this initial rise the systolic and diastolic pressure fell throughout the period of submergence. The diastolic pressure approached zero towards the end of the dive. The pulse pressure was maintained fairly constant during diving except for the initial, transient rise. Upon emersion, both the systolic

and diastolic pressure rose steeply to a value of approximately twice the resting, while the pulse pressure dropped equally abrupt to a very low level. The latter pressure was restored to the pre-diving value in about one minute, whereas the recovery of the systolic and diastolic pressure was not completed until approximately one-half hour after emersion.

During diving, the pressure induced in the large arteries by the ventricular contraction decreased very slowly (Fig. 4), indicating that the rate of emptying of these large blood vessels became extremely retarded.

Blood volume

Two alligators were sacrificed in order to estimate their blood volume by bleeding and washing out the vascular system with oxygenated Frog Ringer's solution. The results are given in Table I below:

Table I. The blood volume of the alligator

Weight	Blood Volume	Blood volume as per cent of body weight
2.7 kg	140 ml	5.1
7.5 kg	410 ml	5.5

Oxygen capacity of alligator blood

Samples of blood were withdrawn from three animals for determinations of the oxygen capacity of alligator blood. The samples were equilibrated with air and analyzed for oxygen. The values obtained are presented in Table II.

Table II. The oxygen capacity of alligator blood in Vol. %

Date	Observed O_2-capacities	Average
8—28—58	10.2; 10.7; 10.1	10.5
9—10—58	10.2; 9.6	9.9
3—12—59	8.2; 7.9	8.1

The content of oxygen and carbon dioxide in the arterial blood

An illustration of the simultaneous changes in the oxygen and the carbon dioxide content of the arterial blood during a dive is presented in Fig. 6. Figs. 7 and 8 give additional data on the amount of oxygen in the arterial blood in two dives of comparable duration. The slopes of the curves are generally steeper in the initial part of the dives than towards the end, showing that oxygen is consumed more rapidly during the earlier stages of the submersions than

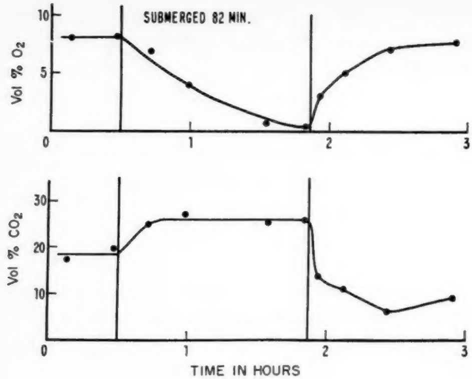

Fig. 6. O_2 and CO_2 concentrations of arterial blood before, during and after diving.

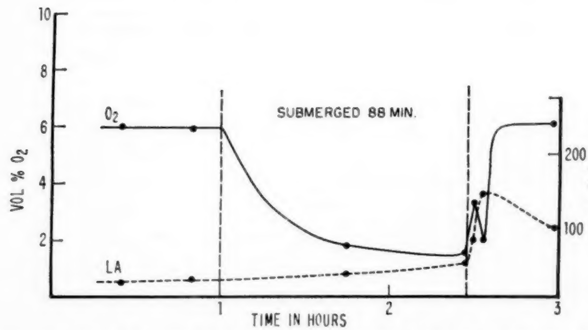

Fig. 7. Concentrations of O_2 and lactic acid in the arterial blood of an alligator before, during and after a quiet dive.

later on. Fig. 6 reveals a rather dramatic situation in which the arterial blood is almost completely reduced just prior to emergence.

The concentration of carbon dioxide increases relatively steeply during the first 20—30 min of the dive, but for the remaining part of the underwater exposure, the rate at which CO_2 enters the blood is markedly diminished. The excessive amount of carbon dioxide in the arterial blood is very rapidly eliminated during the first part of the recovery period. In fact, the CO_2 concentration of the arterial blood will always show sub-normal values during this period, and frequently it may drop to less than 50 % of the level in the pre-diving period.

Lactic acid and pH of the arterial blood

Information regarding the concentration of lactic acid in the arterial blood is furnished in Figs. 7 and 8. In both dives the blood content of lactic acid

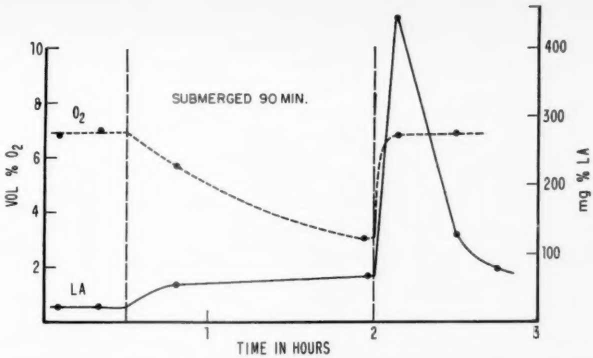

Fig. 8. Concentrations of O_2 and lactic acid in the arterial blood of an alligator before, during and after a strugglesome dive.

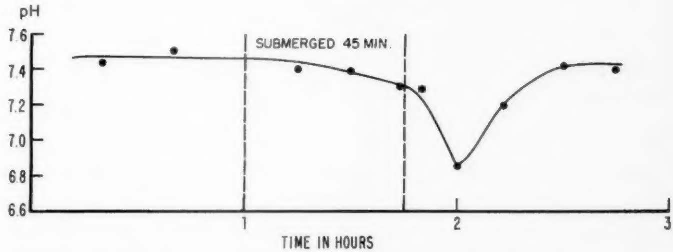

Fig. 9. Variation in pH of arterial blood before, during and after diving.

increased relatively little during submersion. On emersion, however, the blood was rapidly loaded with lactate. The dive illustrated in Fig. 7 was quietly endured, whereas in the other dive, Fig. 8, the animal exhibited bursts of vigorous activity throughout the period of submergence. This, apparently, resulted in a very high level of lactic acid in the arterial blood upon ascent. Contrary to what should have been expected, it appears that the arterial blood is depleted of oxygen at a faster rate in the initial part of the quiet dive than in the other. The arterial blood also contained more oxygen at the end of the strugglesome dive than in the quiet one.

The pH of the arterial blood shows a moderate fall during the dive succeeded by a sudden and very conspicuous drop in the post-dive period (Fig. 9).

Lung volume

The lung volumes of two alligators were measured by means of total body plethysmography. The one, having a body weight of 4.4 kg, was found to have a lung volume of 450 ml. This figure was confirmed in a second experiment.

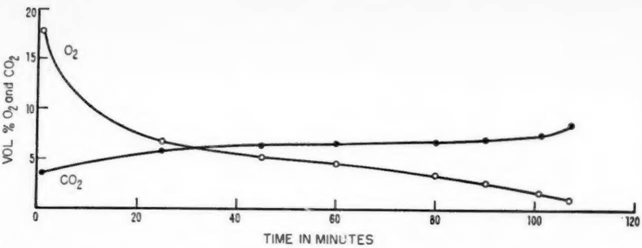

Fig. 10. Changes in the O_2 and CO_2 content of lung air during a prolonged dive.

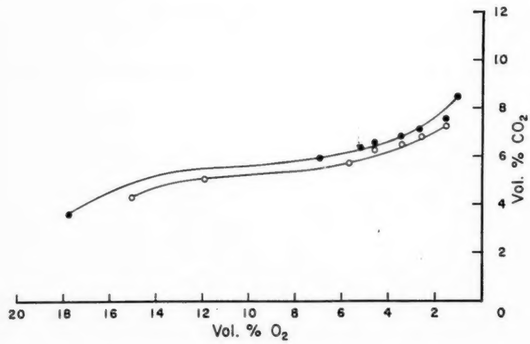

Fig. 11. Composition of lung air during diving plotted in terms of an O_2—CO_2 diagram.

The body weight of the other animal was 3.7 kg. A series of determinations of the lung volume of this animal was carried out. It was estimated to be in the range of 280 to 330 ml. It is very difficult to determine the "normal" inspiratory lung volume of a crocodilian to a fairly narrow range because it varies widely from inspiration to inspiration.

Variations in the composition of the lung air during diving, and the rate of oxygen consumption from this depot

In the beginning of a dive the alligator used oxygen at a fairly rapid rate. As much as one-half of the oxygen in the lung air may be consumed during the first 20 min of a 2 hour dive. During the rest of such a dive, the oxygen of the lung air was used at a much slower rate (Fig. 10). The concentration of CO_2 in the lungs increased slowly throughout the period of submergence. It was found that the content of this gas rises more rapidly during the initial part of the dive than later. At the end of the submersions which lasted until almost all of the oxygen of the lung air was used up, a relatively steep increase in the carbon dioxide concentration was commonly observed. These patterns of the respiratory gas exchange may also be well illustrated in terms of an O_2 — CO_2

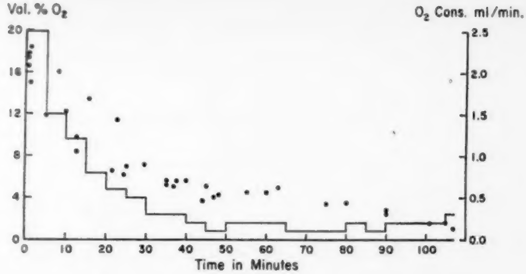

Fig. 12. The depletion (dots) and consumption (columns) of oxygen from the lung air during diving.

diagram like the one of Fig. 11. Here the simultaneous concentrations of oxygen and carbon dioxide have been plotted against each other. The shape of the curves is sigmoid like in all diving animals from which comparable data have been obtained (SCHOLANDER 1940, ANDERSEN 1959 b).

The lung air is the largest oxygen depot in the alligator. The rate of consumption of oxygen from this store during diving would therefore be of great interest. It has already been shown how the concentration of oxygen in the lung air varies with diving time (Fig. 10). If the lung volume did not vary during breath-holding, the actual amount of oxygen in the lungs at any time could easily be calculated from this curve. Due to the shrinkage of the lungs during diving, such a direct calculation is not feasible. It is, however, still possible to quantitate the data from the gas analyses by making the assumption that the amount of nitrogen in the lung air does not change appreciably during diving. This may be expressed by the equation:

$$V_1 \cdot \% N_2' = V_2 \cdot \% N_2''$$

Here V_1 is the lung volume at the start of any interval considered, and $\% N_2'$ is the nitrogen concentration of the lungs in volumes per cent at the same time. V_2 is the lung volume at the end of the interval, and $\% N_2''$ is the corresponding nitrogen concentration of the lungs. The values for $\% N_2$ can be obtained from the gas analyses by subtracting the sum of $\% CO_2$ and $\% O_2$ from 100. Using these values for $\% N_2'$ and $\% N_2''$ in the equation above, V_2 may be calculated provided V_1 is known, and the amount of oxygen contained in the lungs at the start and at the end of any interval subsequently considered. This has been done for intervals of 5 minutes each throughout a diving period lasting for 105 min using an average value for $\% O_2$, $\% CO_2$ and $\% N_2$ in the lung air obtained by utilizing all of the analytical data from the experiments involving such determinations. The results are shown in Fig. 12 where also all the analytical oxygen values are plotted in. The curve showing the average oxygen consumption per minute for every interval of 5 min, has been calculated for an animal with an initial lung volume of 300 ml and at a temperature in

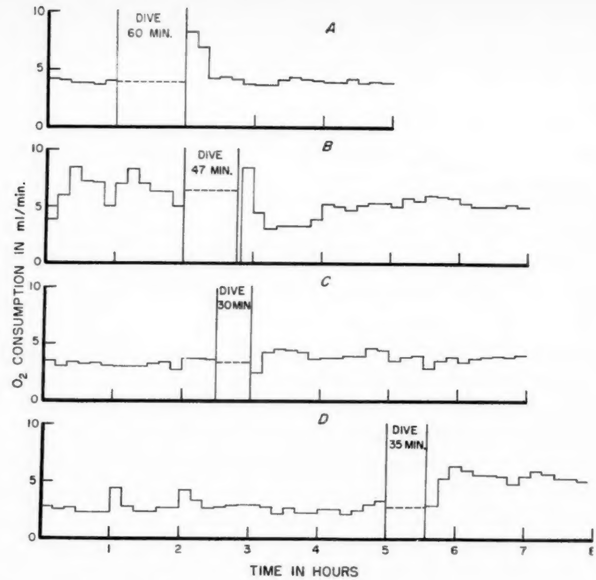

Fig. 13. The pre- and post-dive consumption of O_2 of the alligator.

the range of 21—25° C. It appears that the consumption of oxygen from the lung air would be 0.2—0.4 ml/min during the last 75 min of the dive, *i. e.*, only 3—6 % of the pre-diving oxygen consumption.

Metabolic studies by indirect calorimetry

Pre- and post-dive consumption of oxygen was studied in order to obtain some information on the over-all metabolic rate during diving. A sample o four experiments (Fig. 13 A, B, C and D) has been chosen to represent these experiments. The oxygen consumption is given for intervals of 10 min each. The broken line drawn through the period of submersion in each case is the mean oxygen consumption in the pre-diving period for that particular experiment. In some of the experiments, it was not possible to obtain a very constant rate of oxygen consumption in the pre-diving period. Such an experiment is shown in Fig. 13 B. This was sometimes due to activity on the part of the animal, especially when the experiments were carried out at temperatures above 25° C. In most cases, however, a period of constant oxygen consumption could be recorded if one postponed the dive for several hours.

Upon emersion, the alligator sometimes refused to breathe for several min, *i. e.*, he kept on "diving" after he had access to air. An example is given in

Fig. 13 B. In most of the experiments the excess intake of oxygen after a dive was less than would have been expected if the aerobic and anaerobic processes during diving together maintained the metabolic rate of the pre-diving period (Fig. 13 A, B and C). It happened, however, that the post-dive consumption of oxygen was high, and that it stayed high for hours after the dive was completed.

Discussion

In the present study, it was found that the oxygen depots of the alligator were almost exhausted when the animal had endured experimental diving for about two hours at temperatures around 25° C.

Hence, the cardinal question to be answered in order to explain why the diving animals are able to remain submerged for such a long time, is concerned with the oxygen budget of these animals, since an adequate supply of this gas is indispensible for survival. It seems appropriate therefore to begin the discussion with an evaluation of the oxygen depots of the alligator.

It is generally assumed that the three main stores of oxygen in a diving animal are the lung air, the blood and the muscles. The muscles of the alligator are very pale. This characteristic suggests that they do not contain an appreciable amount of myoglobin, and it is therefore believed that this tissue does not contribute much to the total amount of oxygen stored in the alligator body. The quantity of oxygen present in the lung air and in the blood of the animals can be fairly well estimated from the data reported in the previous section. For the present discussion a specimen weighing about 3 kg will be considered.

A reasonable figure for the lung volume of such an animal would be in the range of 250—300 ml. The extent to which the alligator inflates its lungs during inspiration may, however, vary widely from one respiratory cycle to the next. In order not to under-estimate this oxygen store of the alligator, the larger value will be used here although the animals frequently did reduce this oxygen depot themselves by exhaling a part of their diving air under water.

Immediately after an inspiration the alveolar air may contain roughly 17 Vol % of oxygen. Basing this estimate on the optimal condition that submersion took place just after an inspiration, and that no air was exhaled during the dive, the lung air would constitute a depot of 51 ml of oxygen.

The blood volume of an animal this size would probably be around 150 ml (Table I), and one may assume that the oxygen capacity of the blood would be close to 10 Vol % (Table II).

In case all of this blood was arterialized to full saturation, it would contain 15 ml of oxygen. However, in any vertebrate the greater part of the blood is at any one time in the venous state, and this situation is especially exaggerated in the diving animals. Besides, Figs. 6, 7 and 8 reveal that even in the resting period, the arterial blood is not saturated to more than 60—80 %

of its full capacity. This high degree of unsaturation of the arterial blood may well be due to the fact that the reptilian lung is greatly inferior to the mammalian with respect to facilitation of gas exchange between the alveolar air and the blood. Large areas of the surface of the reptilian lung lack a respiratory epithelium, and are poorly supplied with blood (WOLF 1933). A fair estimate of the amount of oxygen stored in the blood may be obtained if one assumes that 25 % of the blood is arterialized to contain 7 Vol % of oxygen, and that the remaining 75 % is venous with an average oxygen content of 4.5 Vol %. According to these considerations the oxygen depot of the blood of the alligator discussed amounts to about 8 ml, and the total quantity of oxygen available upon submersion can therefore hardly have been much more than 60 ml.

A reasonable figure for the resting oxygen consumption in this animal under the conditions specified would be 4 ml/min. The oxygen stores of this alligator would therefore be completely depleted after about 15 min of quiet diving, provided that the aerobic metabolism remained unchanged at the prediving rate. However, in several experiments, the alligators recovered from dives lasting for 60 to 120 min. Hence, there is an enormous discrepancy between the expected diving time, and the diving performance actually exhibited. This outstanding ability to endure prolonged diving in spite of the insufficiency of the oxygen stores calls for profound metabolic adjustments.

Several authors (DILL and EDWARDS 1931, ELIASSEN 1960) have suggested or implied that the natural divers are able to remain under water for such long periods of time due to an anaerobic shift of the metabolism, thus, making up for the oxygen deficiency by a large increase in the anaerobic processes. However, even if the anaerobic metabolism should constitute the largest source of energy during protracted diving, a certain minimal supply of oxygen will nevertheless be essential in this period. The oxygen stores will have to last until emergence is allowed in order for the animal to survive, for, regardless of any metabolic change, it is clearly impossible to maintain tissues which are susceptible to irreversible damage from oxygen deficiency, such as the heart and especially the brain, in the complete absence of this gas. Therefore, the ability of the alligator and any other of the natural divers to perform prolonged diving and survive must be explained, not simply by a metabolic change towards anaerobiosis, but in terms of mechanisms which operate to make the stores of oxygen last throughout the period of asphyxiation. The development of these mechanisms to a high degree of perfection is probably the characteristic which distinguish the natural divers among the animals, and enable them to endure long-time submersions so successfully. The modifications in physiological function described in the previous section are believed to reflect the operation of the oxygen-saving mechanisms, and in the following these various adjustments and their significance for the diving animal will be considered.

The physiological adjustment to diving most frequently described is the development of a conspicuous bradycardia. This phenomenon is of universal occurrence among the diving animals.

The diving bradycardia is also the most prominent change in the electrocardiogram of the diving alligator, as displayed by the ECG-tracings shown in Fig. 2. After 14 min of diving the time elapsing from one point in the cardiac cycle to the corresponding point in the next was 6.62 sec as compared to 1.30 sec in the pre-diving period. Eighty-seven per cent of this bradycardia was due to a prolongation of the T—Q segment from 0.34 sec to 4.98 sec. As a dive continues the heart rate may drop to 2—3 beats per minute (Fig. 3 A). During this extreme bradycardia the heart spends a progressively increasing part of each cardiac cycle in diastole, *i. e.* it is essentially the T—Q segment of the ECG which becomes further prolonged in the long dives.

It is well known that the heart rate slows down in response to stimulation of the vagal nerves because of a modification in the pacemaker rate. As early as 1899 RICHET found that atropinized or vagotomized ducks show no diving bradycardia. The latter phenomenon was confirmed by LOMBROSO (1913). In the one experiment in which the right vagus was cut in an alligator, a diving bradycardia did not develop. Provided the right vagus was mainly distributed to the sino-auricular junction in this individual as is commonly the case in the mammalian heart, this chance finding tends to support the view that vagal influence also in the alligator is responsible for the slow down of the heart rate during diving.

The length of ventricular systole, as approximated by the Q—T interval, increased 51 % from 0.98 to 1.50 sec during the same time. Because of the rate-dependency of the Q—T interval (BAZETT 1920), the possibility existed that the protraction of this interval resulted from bradycardia only. However, the corresponding $Q—T_c = QT/RR$ interval was found to be more than 25 % increased over the pre-diving value. This means that the slowing of the ventricular systole is due to other factors as well. Because no direct experimental evidence is available, it is at the present time impossible to decide which other conditions contribute to this slowing of the ventricular systole, but it may be a reasonable assumption to attribute it to changes in the nutritional state of the heart, a progressively increasing anoxemia or to the acidosis incurred during diving.

The T-wave also exhibited large modifications during a dive. The amplitude is generally increased during the main part of the submersion, and the wave may occasionally appear diphasic or completely inverted. The T-wave is the most labile part of an ECG, and it is known to be influenced by a wide variety of stimuli including postural changes and changes in the metabolic conditions.

In the literature bearing on the physiology of diving, the bradycardia is generally recognized to be one of the main adjustments to prolonged sub-

mersions, and it is frequently regarded to be of great importance for a successful endurance of such conditions. LOMBROSO (1913), to the contrary reported that vagotomized ducks were still able to dive well. Unfortunately, his paper does not give sufficient data on the diving times endured by the animals to form an opinion of the validity of his conclusion. However, the significance of the low heart rate for the ability to perform prolonged diving does not seem quite clear. Obviously the oxygen consumption of the heart itself will decrease when the number of contractions per minute becomes reduced with some 90–95 %. This must be assumed to be a significant way of saving oxygen during diving because the heart, as will be discussed below, may be the only muscle which receives oxygen while the animal is submerged. Furthermore, it can be seen from Figs. 10 and 12 that the consumption of oxygen from the lung air is much higher in the initial period of the dive before a low heart rate is established, than it is during the remaining period of the submersion. IRVING *et al.* (1941 b) found in the seal that the earlier the bradycardia develops, the longer time will the animal be able to extend its sources of oxygen. These findings, however, do not necessarily imply that the decrease in the oxygen consumption is simply related to the rapidity with which the bradycardia develops. There are, as will be shown, other circulatory modifications associated with diving which are probably far more important than the bradycardia in terms of oxygen saving mechanisms. Knowledge of the proper stimulus required to induce the diving bradycardia would very likely contribute much towards an understanding of the significance of this modification of physiological function.

Considering the data on the alligator, it appears from Fig. 3 A that the bradycardia shows a gradual development. This was characteristic of all the involuntary submersions from which recordings were made. Very frequently an initial tachycardia was observed in these dives. The delay in the onset of the bradycardia points towards asphyxiation as the stimulus for the decreased heart rate. On the other hand, in most of the free dives, the bradycardia appeared much more rapidly (Fig. 3 B), quite often a low heart rate was established before the asphyxiation could be of significance. This is indicated by the ECG-recordings obtained in the resting state, for during regular breathing the alligator frequently held his breath for intermittent periods of 30 to 90 sec, and during this time no change in the ECG was noticed. In the seal, the bradycardia follows immediately after submersion (SCHOLANDER 1940). These observations may indicate that the bradycardia perhaps is brought about by reflex action in response to the submersion, possibly by a general stimulation of the vagal center.

There remains, however, the possibility that the bradycardia is not at all a primary adjustment to diving, but that it merely reflects and compensates for some other circulatory change which may be the principal, circulatory modification during diving. This alternative explanation of the cause of the

bradycardia will be taken up again during the discussion of the circulatory changes.

In order to serve a physiological purpose, the object of the various adjustments exhibited during prolonged diving must, as has already been emphasized, be to extend the period of time which the limited stores of oxygen would normally last, so that the animal does not perish. A functionally excellent solution of this problem would be to shut off the blood supply to some organs and tissues which are not critically dependent upon a continuous, ample supply of oxygen. This would not only diminish the total consumption of oxygen per unit time markedly, but it would leave the oxygen for the more sensitive parts of the organism. There is strong evidence that such a mechanism does operate in the diving animals.

In Figs. 7 and 8, the concentrations of oxygen and lactic acid in the arterial blood have been illustrated in two dives of comparable length. The dive illustrated in Fig. 7 was quietly endured, whereas in the one shown in Fig. 8, the animal struggled vigorously several times throughout the diving period. As a result of this difference in diving behaviour, the lactic acid concentration of the blood rose to 150 mg % vs. 450 mg % respectively, in the post-diving period. During the dive, however, only a comparatively small increase in the concentration of blood lactate was observed. In both dives the concentration of oxygen in the arterial blood diminished from 6—7 Vol % at the beginning of the dive to approximately 2—3 Vol % just prior to emersion. The loss of oxygen was rapid in the initial part of the dives, and it parallels a similar depletion of oxygen in the lung air (Figs. 10 and 12). However, although the one dive (Fig. 8) was so uneasily endured, as judged by the struggling of the animal, the rate with which the oxygen was lost from the blood was no greater than in the quiet one. At the end of the dive the animal which showed the high degree of activity during diving displayed a larger concentration of oxygen in the arterial blood than the other. These two findings show that there is little, if any, exchange of oxygen and metabolites between the muscles and the circulating blood during diving, *i. e.* the muscular tissue is not perfused. The constriction of small arteries in the thigh was frequently observed during diving in the experiments in which the femoral artery was exposed. These findings fit well with the observation of SCHOLANDER *et al.* (1942) that the myoglobin of seal muscles is completely reduced at an early stage of a dive when the arterial blood is still half saturated. Considering that the myoglobin has a greater affinity for oxygen than hemoglobin has, the only reasonable conclusion to be drawn from these findings is again that the muscles were not perfused while the animal was submerged. When one appreciates the large mass of muscular tissue and its high rate of oxygen consumption when active, the shut off of the muscular circulation must be recognized to constitute a most beneficial regulation for the purpose of sparing oxygen so that a submersion may be safely extended. Moreover, it is very probable that the

vasoconstriction involves other circulatory beds than the muscular one, for Irving *et al.* (1942) reported that the diameter of the smaller arteries and veins of the mesentery gradually diminished during diving until they became quite bloodless in appearance, and the color of the gut itself showed cyanosis of this organ shortly after submergence. Such a widespread vasoconstriction implies a general increase in peripheral resistance. This is suggested by the increasingly gentle slope of the descending limb of the arterial blood pressure curves in Fig. 4, which relates to the diastolic phase of the cardiac cycle, because this observation indicates a retarded rate of emptying of the reservoir of the large arteries. A similar picture could, of course, have been obtained if the stroke volume increased much in order to keep the cardiac output constant. This is less likely, however, since it calls for a dilatation of the heart up to 15 times.

As a result of the suggested increase in the peripheral resistance, one would expect a marked rise in the arterial blood pressure as soon as the vessels of the various vascular beds involved start contricting. That this actually happened is clearly demonstrated in Fig. 5. The systolic, diastolic and pulse pressures were consistently observed to increase 60—70 % above resting level. This makes it possible that the bradycardia is reflexly initiated in response to the circulatory adjustments leading to this elevation in the arterial blood pressure. If this is so, one is left with the puzzling fact that in spite of the advancing fall in arterial blood pressure throughout the remaining part of the dive, the bradycardia persists or is even developed further (Fig. 3 A). There is one plausible explanation for this phenomenon, namely, that the bradycardia initially established either by the submersion itself or in response to the elevated pressures, is maintained, and in some cases further developed by an increasing degree of asphyxiation, but the complexity of the situation is such that at the present time a final answer to the cause and maintenance of the diving bradycardia cannot be provided.

The shut off of the perfusion to the muscular tissues persists evidently throughout the dive, as is apparent from the lactic acid picture of the arterial blood. This calls for an explanation because it is well known that accumulation of lactic acid or carbon dioxide, decrease in the oxygen tension or a fall in pH tend to relax the arterioles. It is necessary to postulate then, that the effect of these local vasodilators is overcome by some other factor, presumably of nervous or endocrine character. How this is brought about is not known.

Besides reserving the oxygen stores for the brain and the heart, another principle required for the safe extension of under-water exposure would be the ability to utilize the oxygen stores to a very high degree and still maintain a state of "useful consciousness". Useful consciousness has been defined to mean "that state in which the individual remains attentive and is able to perform useful or purposeful acts" (Hall 1949). In the crocodilians it seems likely from their method of catching prey that this feature must be of direct

value, for these animals are known to grasp their victim, pull him under water to drown him, and then again surface in order to feed.

Whereas man usually loses consciousness if the partial pressure of oxygen in the inspired air falls below 60 mm Hg, which corresponds to roughly 8 Vol % at sea level, the alligator stays alert and will immediately surface when this is made possible, after a dive like the one in Fig. 10, where the lungs contained only 1 Vol % of O_2, *i. e.* the partial pressure was only 7 mm Hg STPD. At first thought one would expect to see this high degree of oxygen utilization reflected in a corresponding increase in the CO_2 concentration of the lung air, and in corresponding changes in the content of oxygen and carbon dioxide in the arterial blood. The arterial O_2 tension fell in accordance with the diminishing content of oxygen in the lung air as seen by comparing Fig. 10 with Figs. 6, 7 and 8. However, the rise in the amount of CO_2 in the lung air is always low during diving relative to the drop of the oxygen content, and the *RQ* for the lung air is for the main part of the dive less than 0.5 which is shown in Fig. 11 where the corresponding concentrations of oxygen and carbon dioxide have been plotted against each other. This situation is still further exaggerated in the blood (Fig. 6) where it seems like the concentration of CO_2 does not at all increase after the initial rise during the first 20 minutes of the dive. Here it ought to be mentioned that whereas the analyzer used for the gas analyses easily permits detection of variations of $\pm$ 0.02 %, the instrument used for the blood carbon dioxide is limited in accuracy to $\pm$ 1 %. Anyhow, the rather slow increase in the carbon dioxide content of the arterial blood and the alveolar air should not surprise us considering that the CO_2 can be stored and buffered in the living organism. In the first place, the diving alligator has only a small amount of stored oxygen from which carbon dioxide can be produced, and a large fraction of the CO_2 formed is probably buffered in the tissues.

The hypothesis that a significant fraction of the carbon dioxide formed is buffered in the tissues during submersions has been experimentally verified in a qualitative way in an earlier study of the diving duck (Andersen 1959 b), and there is no obvious reason to doubt the applicability of the observations discussed above to the alligator.

In accordance with the lactic acid picture, the pH of the arterial blood showed its largest drop in the recovery period (Fig. 9). There is a concomitant decrease in the blood content of CO_2 to less than 50 % of the normal value which may be seen from Fig. 6. This is probably due to an expulsion of carbon dioxide from the blood by the lactic acid.

The physiological significance of the functional adjustments discussed in the previous sections is to delay the exhaustion of the limited oxygen stores of the diver. It remains to be evaluated how these adjustments influence the rate of energy metabolism during submergence. The patterns of the aerobic metabolism during diving are revealed by the oxygen consumption from the lung air. In Fig. 12, the average oxygen consumption in ml/min from this store

is shown for successive period of 5 min each throughout a dive lasting for 105 min. During the last 65 min of this dive, the average consumption of oxygen from this store was 0.3 ml/min, or only about 5 % of the corresponding figure for the pre-diving period at a temperature of 25° C. If the anaerobic processes are going to make up for the deficit they will have to work at an extremely high rate. The total metabolic rate during diving is most easily revealed by the study of the oxygen debt incurred by comparing the pre- and post-dive oxygen consumptions. A sample of four of these experiments has been reported in Fig. 13 A, B, C and D. In most of the experiments, the post-dive excess intake of oxygen did not cover the oxygen debt during diving. The one extreme is pictured in Fig. 13 C where there is only a very slight excess intake of oxygen. On the other hand, it occasionally happened that the O_2 consumption after emersion was enormous (Fig. 13 D). In such cases, however, it seemed like the O_2 consumption was started at an entirely new level, and that it was maintained continually at this high rate for several hours. In the experiments in which the excess uptake of oxygen after the dive did not cover the anticipated oxygen debt, the results inescapably imply a very marked decrease in the total rate of energy metabolism during diving. This finding is in perfect agreement with the observations on the homoiothermic, diving animals studied (SCHOLANDER 1940, ANDERSEN 1959 a).

The work reported in this paper was carried out in the laboratory of Dr. P. F. SCHOLANDER at Scripps Institution of Oceanography, University of California, La Jolla, California. I am very deeply indebted to Professor SCHOLANDER for his enthusiastic and critical supervision of my work. At the University of Pennsylvania, Philadelphia, I have benefited greatly from many discussions of the problem with Drs. H. T. HAMMEL and W. S. YAMAMOTO.

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From The Zoophysiological Institute, University of Lund, Sweden

Ribonucleic Acid and Acid-Soluble Nucleotides of the Early Chick Blastoderm

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Abstract

EMANUELSSON, H. *Ribonucleic acid and acid-soluble nucleotides of the early chick blastoderm.* Acta physiol. scand. 1961. *53.* 46—57.—Previous analyses of the nucleic acid content of the embryo area in early chick blastoderms have been extended to comprise also the area opaca. The RNA-variations in the latter are reminiscent of those in the embryo area, but from the 10-hour-stage on their RNA/DNA-ratios are characteristically different. The RNA of the early blastoderm has been analyzed for its nucleotide-composition and also a limited analysis of the acid-soluble nucleotides has been undertaken. The last-mentioned fraction is especially distinguished by a high proportion of uracil-nucleotides. To the account is added an analysis of the content of nucleic acids and acid-soluble nucleotides in the unincubated hen's egg.

In a previous investigation (EMANUELSSON 1958), the author has shown that during the early development of the chick embryo there occur considerable variations in the concentration of ribonucleic acid and nucleotides in the central part of the blastoderm, the embryo area. These striking fluctuations will naturally give rise to the question of whether similar conditions also prevail in the peripheral part of the blastoderm, i. e., the area opaca. Furthermore, one may ask whether the recorded variations are purely quantitative; the radical morphologic changes in the embryo during the investigated period of development suggest that qualitative changes may be involved too. Information bearing just upon these problems is given in the present paper together with

details about the nucleotide content of the early blastoderm. The account is supplemented with data about the occurrence of nucleic acids and nucleotides in the extra-embryonal parts of the hen's egg, the egg-white and the yolk.

Material and Methods

The experimental material was newly laid hen's eggs (White Leghorn, pure breed). Incubation of the eggs was carried out in a thermostatically controlled incubator at 37.5° C.

When otherwise not stated the data for egg-white and yolk refer to conditions in the unincubated egg. Analyses on the white yolk were made on material collected from eggs incubated for 24 hours in which the white yolk is more easily separated from the blastoderm than in the unincubated egg. Thus the white yolk, referred to in the present paper, is more exactly that fraction of the white yolk which is contained in the "nucleus of Pander".

Isolation of the ribonucleic acid (RNA) in the area opaca, egg-white and yolk was carried out as earlier described (Emanuelsson 1958), by extraction with hot 10 per cent NaCl; after hydrolysis in N NaOH the absorption of the RNA-nucleotides was measured in a spectrophotometer at 260 mμ and the values were corrected for the higher extinction shown by the hydrolyzed material as compared with the unhydrolyzed RNA. When analyzing egg-white and yolk it proved necessary first to purify the RNA-hydrolysate by the norite method (see below) before the readings were made.

Determination of the desoxyribonucleic acid (DNA) of the area opaca was also made as earlier described; i. e., after extraction with 10 per cent NaCl and removal of RNA by hydrolysis in N KOH the absorption of alcohol-precipitated DNA was determined spectrophotometrically.

Estimation of the DNA in egg-white and yolk was made according to the Schmidt-Thannhauser method using the diphenylamine test, as quoted by Dische (1955). Owing to inevitable interference from other components in the trichloroacetic acid extract with the test, the latter was supplemented with chromatographic analyses of the nucleic acid derivatives in the extract.

Acid-soluble nucleotides were extracted by homogenization of the tissue material together with the same volume 10 per cent ice-cold trichloroacetic acid. After repeated extraction with 5 per cent trichloroacetic acid the combined extracts as well as the precipitate intended for RNA-analysis were shaken 6 times with ether to remove the trichloroacetic acid. However, before this treatment extracts from yolk and egg-white had first to be filtered through Hyflo Supercel. The ether was removed by aeration, whereupon the nucleotides were purified by adsorption on norite at pH1. After washing the norite with distilled water the nucleotides were released by shaking the norite 5 separate times with a solution of 50 per cent ethanol containing 3 ml conc. ammonia per 100 ml. The method gives a satisfactory yield, although it is known that of the nucleotides DPN and TPN are not released quantitatively.

The purified nucleotides were usually directly hydrolyzed in N HCl and the chromatographed in isopropanol-concentrated HCl-water (97:25:28). The method makes it possible to separate the material into groups of nucleotides with different mother substances (bases) and permits estimation of the total amount of nucleotide-material belonging to each group.

When chromatographing the purified extract of acid-soluble nucleotides troublesome interference with the cytosine-derivatives was experienced from unidentified substances, ninhydrinpositive and with absorption-maximum around 275 mμ. The values, recorded for the cytosine-nucleotides must therefore be considered as very approximate.

Owing to the small amounts of nucleotides usually available a more detailed qualitative and quantitative analysis of the members of each group was considered too uncertain and was consequently omitted. In those cases when identification of a separate nucleotide directly was required the latter was isolated and identified by chromatographing the sample two-dimensionally in propanol-concentrated ammonia-water (60:30:10) and saturated ammonium sulphate -isopropanol-water (72:2:19) respectively.

All chromatograms in the present investigation have been of the ascending type and were made on Whatman No. 1 papers. When possible they have always included some sort of test-substance (usually RNA-hydrolysate) which was run parallel with the sample to be analyzed. In the chromatograms detection of the spots was made in UV-light (max. emission at 260 mμ). The spots were eluted over night with 0.1 N HCl, the different substances being conclusively identified and estimated by reading the eluate in spectrophotometer.

The relative proportions of the different nucleotide groups from the trichloroacetic acid extract were in fairly good agreement with that found earlier, when — as described in an earlier publication — lanthanum acetate was used to precipitate the nucleotides.

The nucleotide analyses on RNA refer to RNA hydrolysed in N KOH at 37° C for 1 hour. The alkaline hydrolysate was neutralized with perchloric acid and after removal of DNA by precipitation with acidified alcohol in final concentration of 70 per cent the sample was partly evaporated in vacuo, hydrolyzed in N HCl and chromatographed in isopropanol-concentrated HCl-water as described above. In most instances it proved necessary first to purify the RNA-nucleotides by norite adsorption (see above) before the hydrolysis in HCl. Detection, identification and estimation of the products of hydrolysis in the chromatograms were performed as described for the acid-soluble nucleotides.

Sugar analysis of the white yolk was made according to the phloroglucinol method of v. Euler and Hahn (1946). The absorption of the resulting colored solution was read in spectrophotometer at 422 mμ together with glucose standards.

Nitrogen analysis of the white yolk was made according to Boissonnas and Haselbach (1953).

The cultivation of embryo explants has been described previously (Emanuelsson 1958). Here should only be mentioned that the culture medium consists simply of chicken-Ringer to which has been added agar-agar + glucose. After 24 hours' incubation on this medium explants show poor growth but excellent differentiation.

Results

RNA of the Area Opaca

Analysis of the whole chick blastoderm reveals that not only the embryo area but also the area opaca around it is subjected to considerable changes in the cellular concentration of RNA.

For the embryo area it was earlier demonstrated that during the first 60 hours of development the RNA/DNA-quotient ranges between 2,5 and 10.

It is now found (after analysis of the area opaca from various developmental stages between 0 and 60 hours of incubation) that also within the area opaca there exists a similar variation, the values ranging between 3 and 10. The low values (3—4) for the latter region are attained at about the 10-hour and the 20-hour-stage respectively. At other stages the values are mostly high (around 8), and it is especially noted that from the 35-hour-stage until the 60-hour-stage the RNA/DNA-level lies fixed at the value 8. On the two occasions when

Table I. RNA composition of early chick blastoderms

Stage of development	Part of blastoderm	Molar proportions					$\frac{G+U}{A+C}$	$\frac{Pu}{Py}$
		A[1]	G	C	U	G/C		
0 hours..	Whole blastoderm......	10.0	12.1	10.8	7.0	1.12	0.92	1.24
18 hours..	Embryo area..........	10.0	14.7	11.0	8.7	1.34	1.11	1.11
18 hours[2]	Embryo area..........	10.0	10.2	10.6	9.4	0.96	0.94	1.01
	Area opaca	10.0	15.6	15.7	15.6	1.0	1.22	0.82
24 hours..	Embryo area..........	10.0	15.5	14.0	12.0	1.11	1.15	0.98
	Area opaca............	10.0	15.2	13.4	11.6	1.13	1.15	1.01
18+24 hours[2].	Explanted embryo area..	10.0	14.5	13.3	11.6	1.09	1.12	0.98
48 hours..	Embryo area..........	10.0	16.5	13.2	9.0	1.25	1.10	1.19
	Area opaca............	10.0	15.8	11.0	8.1	1.44	1.14	1.35
72 hours..	Whole blastoderm......	10.0	18.3	12.3	10.5	1.49	1.29	1.26

[1] Abbreviations used: A = adenylic acid, G = guanylic acid, C = cytidylic acid, U = uridylic acid, Pu = purines, Py = pyrimidines

[2] Cooled for 5 days at + 2° C

[3] 18-hour-embryos cultivated for 24 hours in vitro

the quotient drops to its lowest values for the area opaca, marked decreases are also met with in the embryo region. Otherwise it is apparent that during the investigated period of development the two parts of the blastoderm show fundamentally different trends of development as regards the RNA/DNA-quotient: in the embryo region it is gradually decreasing to a low level, in the area opaca it is finally stabilized at a high level. From the 10-hour-stage on this difference is quite obvious.

No attempts have yet been made by the author to estimate the amount of DNA per cell in the area opaca. However, as it has been found for the embryo area that the DNA-content during the first 24 hours will reach values twice the amount which is recorded after that period (EMANUELSSON 1961), it is not excluded that at the same stages of development there is a similar variation in DNA-content in the area opaca too. Consequently it cannot be definitely argued that the RNA/DNA-quotient of the area opaca truly reflects the average amount of RNA per cell, but even if it were calculated with the aforesaid DNA-changes in this region too, the RNA-variation during the first 30 hours would nevertheless be appreciable.

Besides a normal variation in the RNA/DNA-quotient in the blastoderm cells it is found that for early stages (10-20 hours of development) interruption of incubation when already in progress will soon lead to a decrease of the same quotient both in the embryo region and the area opaca. This condition was observed when investigating the formation of anidian blastoderms, i. e., blastoderms in which the embryo area is more or less degenerated but the margin (area opaca) still continues to develop, essentially normally. This phenomenon may be

evoked if during the earliest development the eggs are cooled down and kept at —3° C for some days and then are reincubated at normal temperature (+37,5° C) (Grodzínski 1934). A less drastic treatment, involving cooling for some days at + 2° C, has been found to cause morphologic disturbances of only a minor part of the treated embryos during the continued incubation. The cytologic disturbances, however, are common in all the embryos, mainly reflected in abnormally extended anaphase chromosomes.

Now biochemical analysis of this embryo material (18 hour blastoderms, cooled for 5 days at + 2° C) shows that RNA/DNA-quotients as compared with the initial conditions before cooling have decreased with 29,5 per cent (embryo area) and 24 per cent (area opaca). Even if about half this decrease must be ascribed to DNA-synthesis taking place during cooling of the eggs, the other half undubitably represents a real loss of RNA, which may be estimated to between 5 and 10 per cent of the total RNA-content in each region. In the embryo area a 14 per cent increase of the normal level of free nucleotides was simultaneously registered. Qualitative changes of RNA have also been recorded for the cooled blastoderms (Table I).

Nucleotide Composition of RNA from Early Chick Blastoderms

The analytic data are assembled in Table 1. For each stage they represent average values from 4—6 separate analyses, each comprising material from 50—200 blastoderms. They have all been in close agreement.

In the unicubated blastoderm there are still no differences in RNA-composition between the central embryo area and the peripheral area opaca. It is to be noted, however, that at this stage, when development is at a standstill, even if mitotic activity is not altogether lacking the proportions between the RNA-nucleotides are markedly different from what are found in more advanced stages in active growth.

When incubation has started a slight but scarcely significant difference in RNA-composition between the two actual regions of the blastoderm is indicated after 24 hours. After an additional 24 hours the difference is clearly pronounced, the Pu/Py ratios being markedly dissimilar. A common feature for both regions during the course of development is the gradually increasing proportion of guanylic acid in the RNA.

Closer inspection of the found nucleotide ratios will disclose certain regularities in their variation, which reasonably should have connection with actual conditions of cell-multiplication prevailing in the analyzed regions. In the present case it seems to the author that of the molar relationships included, the G/C-ratio, i. e., the relation between guanylic and cytidylic acid, most strikingly reflects the mitotic activity in the investigated material. Thus high values of this ratio apparently correspond to high mitotic activity, whereas low values denote the reverse condition. The observed connection is clearly indicated

Table II. Acid-soluble nucleotides of early chick blastoderms

Stage of development	Part of blastoderm	Nucleotide composition (as moles per 100 moles nucleotide)			
		A[1]	G	C	U
0 hours	Whole blastoderm	47.4	8.0	10.4	34.2
24 hours	Embryo area	45.0	8.3	14.7	32.0
	Whole blastoderm	43.2	8.7	5.7	42.4
48 hours	Whole blastoderm	63.0	16.9	8.2	11.9
72 hours	Area opaca	52.6	11.8	6.5	29.1
	Whole blastoderm	56.3	19.5	7.3	16.9

[1] Abbreviations used: A = adenine nucleotides, G = guanine nucleotides, C = cytosine nucleotides, U = uracil nucleotides

by the analytic values obtained from ordinary 18-hour-embryos and 18-hour-embryos cultivated in vitro on a poor culture medium totally lacking in proteins. The former embryos show a high mitotic index, higher, e. g., than in the succeeding more advanced 24-hour-embryo, whereas in the explants the mitotic activity is low after 24 hours' incubation. Nevertheless a differentiation has occurred in them which is nearly equivalent to that in normal embryos of corresponding age.

Except for the 72-hour-blastoderms the investigated material has shown a composition of the RNA which is in fairly good agreement with "Chargaff's rule", i. e., the bases with 6-amino groups and those with 6-keto groups occur in approximatively equal amounts (Elson and Chargaff 1955).

Acid-Soluble Nucleotides of the Early Chick Blastoderm

The author has earlier reported the occurrence of a varying level of free nucleotides in the early chick embryo. In the present investigation some quantitative relationships for these nucleotides are presented, which furnish at least a rough picture of conditions prevailing in the early chick blastoderm. Paper chromatography has in all cases been the method of analysis. It is obvious that ion-exchange chromatography would have been preferable, but owing to the difficulties in collecting sufficient material for that type of analysis it had to be left out of consideration.

Analysis of trichloroacetic acid extracts from early blastoderms reveals the occurrence in them of nucleotides which are derivatives of adenine, guanine, cytosine and uracil. The relative proportions of these are given in Table II. Each stage is represented by at least 4 separate determinations, which all have been in good agreement. In fact traces of thymine derivatives were also observed in the extracts from the 24-hour-blastoderms.

When reading the table it should be observed that the proportion of cytosine

derivatives stated here is approximative only and presumably too high. Furthermore, it should be stated that it has been impossible to obtain the early blastoderms free from adhering yolk granula. These yolk granula together with already absorbed, intracellular yolk material will obviously constitute a factor of dilution. As will be shown below, the yolk granula are characterized by a comparatively high amount of uracil nucleotides, so there is reason to suppose that the high values for the uracil derivates, stated in Table II, to a certain extent refer to extra- and intra- cellular yolk (and presumably also absorbed egg-white) material. If that assumption holds true, the values in Table II suggest higher relative amount of yolk material in the earliest developmental stages, moreover they would indicate more yolk granula in the area opaca than in the embryo area. Both conditions were confirmed on cytologic examination.

Of the remaining groups of nucleotides included in The Table II, namely, the adenine derivatives and the guanine derivatives, the former are quantitatively dominating. The absolute amounts are of less interest in the present case, bearing in mind the possible variations in the amount of yolk present in early blastoderms, yet it appears that for both the 48-hour- and the 72-hour-stage the concentration of adenine nucleotides is comparatively similar, corresponding to c. 110 μM adenine/100 g wet weight. For earlier stages the concentration of adenine derivatives is apparently higher.

As for the mutual relation between the two last-mentioned groups of nucleotides it is of interest to record that the lowest proportion of guanine derivatives is met with in the 0-hour-blastoderm, the highest in the 48-hour-blastoderm. Thus a resemblance to the variations of the G/A-ratio for RNA from the same developmental stages is evident. The analytic data for normal 18-hour embryos and 18-hour embryos cultivated in vitro are partly incomplete, but they have nevertheless shown that also these stages follow the same pattern with respect to the acid-soluble adenine and guanine derivatives.

Nucleic Acid and Acid-Soluble Nucleotides in Egg-White and Yolk

When dealing with problems connected with the synthesis of nucleic acids in the developing chick embryo it should not be forgotten that in the extra-embryonal parts of the hen's egg there is in fact a significant amount of nucleic acids and nucleotides. The role of these in the development of the chick embryo is, however, still poorly understood.

Fraenkel—Conrat et al. (1951) were the first to establish definitely the occurrence of DNA-protein in the egg-white of the hen's egg. Later analyses by Solomon (1957) have demonstrated the occurrence of DNA and RNA, both in egg-white and yolk. As owing to high protein-contamination in combination with large extract-volumes, the previous analyses of the present author failed to prove the definite existence of DNA in the yolk of the hen's egg, a reinvestigation based on other methods of analysis has been made of the nucleic acid content of the egg-white and the yolk. The values given in Table III were arrived at for

Table III. Nucleic acid content and acid-soluble nucleotides of the unincubated Hen's egg

Average weights: egg-white .... 35 g			*Acid-soluble nucleotides*			
yolk .... 17 g				Nucleotide composition (as moles per 100 moles nucleotide)		
Nucleic acid content	DNA	RNA		A[1]	G	U
Egg-white	160 μg	250 μg	Egg-white .	8.1	27.8	64.1
Yolk (blastoderm removed)	50 μg	60 μg	Yolk	9.9	24.1	66.0

Absolute amounts of uracil nucleotides: egg-white c. 1.5 μM/100 g, yolk c. 0.6 μM/100 g

[1] Abbreviations used: see Table II

unincubated hen's eggs. The figures are in tolerable agreement with Solomon's results, but they are somewhat lower throughout. Besides an obvious variation in the DNA-content of the yolk ($\pm$ 20 per cent) of the investigated eggs, it was found that the DNA is not uniformly dispersed but occurs in notably higher concentration (10—15 times higher) in the white yolk, located directly under the developing blastoderm. Other analyses further revealed that calculated per unit of nitrogen the sugar content of this white yolk is appreciably higher than in the yellow yolk (1.07 μg/μg nitrogen against 0,76 μg/μg nitrogen, in both cases calculated as glucose).

Concerning the DNA of the entire egg-white the present author has registered an apparently significant decrease already during the first day of incubation. The decrease which is slightly higher than that recorded during the second day of incubation amounts to 10 per cent of the whole DNA-content.

Investigation of the nucleotide content of the extraembryonal parts of the hen's egg has been made on similar lines as for the blastoderms, i. e., as an analysis of the proportions of the principal types of nucleotides. The values are given in Table III. Cytosine nucleotides are not included in the table as the recorded amounts are quite insignificant.

Separate analysis of the nucleotide content of the white yolk indicates much higher concentration of adenine nucleotides than in the yellow yolk and a dominance of adenine nucleotides over guanine nucleotides which is quite different from the conditions which prevail in the yolk as a whole. The greater part of the adenine-nucleotide-fraction is composed of ATP.

Discussion

Changes of the RNA/DNA-quotient in the area opaca of the early chick blastoderm at about the same time as the occurrence of rather similar changes in the embryo area bear evidence of a fairly similar character of growth in both the regions. From the 10-hour-stage on, however, it is clear that the ratio in question

is constantly higher in the area opaca. This condition together with the fact that the periods with decreased RNA/DNA-ratios apparently are not quite synchronous for the two areas will explain the fact that already during the first 30 hours of incubation important ratio-differences may occur at certain stages, e. g., the 18-hour-stage. A more decided deviation is otherwise not evident until the second day of incubation.

For the 18-hour-stage the ratios in fact indicate a RNA concentration in the area opaca about twice as high as that in the rest of the blastoderm. It seems probable that the different sensitivity towards external disturbances which at this stage is displayed by the two regions is connected with these ratio-differences. From the author's experiments it is obvious that factors which affect RNA-synthesis of the embryo cells, e. g., extreme temperatures, added steroids etc., have a more pronounced effect upon the embryo area than upon the area opaca. The resulting effect may then be either morphologic disturbances or intracellular changes, visible as chromosome disturbances.

The lack of a functioning vascular system for the rapidly thickening embryonal area will possibly place the nutrition — and with that also the ability to RNA synthesis — in a more exposed position for that region; in the more peripheral parts there is still intimate contact with the underlying food reserves. The decrease of the RNA/DNA-ratio in blastoderms in which incubation is interrupted by cooling indicates RNA-deficiency as a plausible cause in the formation of anidian blastoderms. In these the degenerative changes — as mentioned — affect the embryo region but not the area opaca, at least not the outer part thereof. Even if now both the actual regions of the chick blastoderm will show a RNA-decrease during cooling, it seems probable that just the cells of the embryo area with their markedly lower RNA/DNA-ratio, will sooner reach a relation between RNA and DNA which is no longer consistent with normal growth and differentiation at the stage in question.

The dominance of the adenine and cytosine components, shown by the RNAs of the embryo area and the area opaca, is apparently a common feature for RNA of animal tissues (Magasanik 1955). On the whole the ratios for the components reflect about the same pattern which according to the latter author is found for chicken liver, namely A : G : C : U = 10.0 : 17.1 : 13.6 : 10.6. The ratios reported for RNA from 48- and 72-hours-blastoderms are, however, markedly different from values obtained for RNA isolated from the area opaca of 40—60-hour-blastoderms by Kirby's phenol method (Emanuelsson 1960). The following ratios were then found: A : G : C : U = 10.0 : 22.8 : 16.3 : 15.8. Even if the present analyses do not refer to exactly that stage, a comparison may yet be drawn which shows that the last mentioned values indicated a higher G/A-ratio (2.28 against 1.58) and a lower Pu/Py-ratio (1.02 against 1.35) than found here for practically equivalent material.

Now it is a well-known fact that RNA-preparations isolated by different procedures vary greatly in composition (Magasanik 1955) and it has just been

shown by Yamana and Sibatani (1960) and Sibatani et al. (1960) that phenol-released RNA does not represent the total RNA-content of a tissue but a major fraction thereof, metabolically little active and with a high guanylic acid content. It is presumably synthesized at about the same rate as DNA, i. e., duplicated only once during the mitotic cycle. Left behind in the phenol phase — and thus actually missed in the isolation procedure — there remains a metabolically active RNA-fraction with low guanylic acid content.

The varying guanylic acid content of the RNA recorded for the blastoderms here in Table I seems quite consistent with the assumption of two metabolically different classes of RNA. The increasing guanylic acid ratio of the more advanced stages indicates a diminished proportion of metabolically active RNA which is just to be expected, bearing in mind the decreasing rate of cell-multiplication and the proceeding cell-differentation.

Also the variations of the G/C-ratio with the actual state of cell-multiplication in the embryo are now more clarified, but it is obvious that a definite evaluation of this ratio cannot be made until complete data about the composition of the two classes of RNA are available. Yet it will seem from the analytic values reported by Sibatani et al. (1960) that the higher G/C-ratio is shown by the metabolically less active RNA.

For the area opaca an interpretation of the present analytic values in accordance with the suggestions proposed above would suggest higher mitotic activity and higher proportion of metabolically active RNA than in the adhering embryo area. It seems very likely that this is in conformity with the actual conditions.

The absolute amount of acid-soluble nucleotides and the prevailing relations between adenine and guanine nucleotides found in the present investigation correspond fairly well to data arrived at for rapidly growing animal tissues (Saukkonen 1956). The high proportion of uracil derivatives is, however, a special characteristic of the early blastoderm and is reasonably associated with the presence of uracil derivatives in the egg-white and the yolk. In 15-day-old chick embryos the proportion of uracil derivatives among the acid-soluble nucleotides is apparently much lower (Lu and Winnick 1955). It is generally acknowledged that the avian liver is rich in uracil compounds. However, in the present case it should be especially pointed out that Strominger (1954, 1955) has isolated from the hen's oviduct several types of uridine and guanosine nucleotides, i. e., representatives of the two groups, which dominate in the egg-white and the yolk. In the chromatograms of the oviduct extract the former group was dominating, it amounted to c. 0.5 μM/g tissue.

Our present knowledge of the role of the uridine nucleotides shows that they are of great importance in transformation processes between different sugar compounds and in synthesis of polysaccharides. It seems justifiable to assume a close connection between the high amount of uracil derivatives in early blasto-

derms and the established condition (SPRATT 1951), that carbohydrates is the main source of energy for the chick embryo during the first four days of incubation. In this connection should also be emphasized the peculiarity that the very structure of the yolk — with the white yolk close up to the blastoderm — has obviously secured the embryo a direct supply of energy-rich phosphate and carbohydrate for the earliest development.

The intense protein-synthesis in the early blastoderm explains the high amount of cytosine- and guanine-nucleotides, as according to our present knowledge (HOAGLAND 1960), both types play an essential rôle in protein synthesis. The observed similarities in variation between RNA and the nucleotide fraction with respect to the adenine/guanine-ratio permits the assumption of a close connexion between the acid-soluble nucleotides and RNA. That nucleotides may act as precursors in nucleic acid synthesis is actually verified in recent investigations, cf. e. g. KHORANA (1960).

At the same time it should be realized that part of the nucleotides in the blastoderm evidently emanate from nucleic acid degradation. Even if decomposition of nucleic acids of the embryo during the isolation procedures is not very likely, the very presence of nucleic acids both in egg-white and yolk and the evident ribonuclease-activity in the blastoderm — indicated by the RNA-decrease in the cooled embryos — favors such a view. Also the rapid decrease of the DNA of the egg-white during the first day of incubation and the observation of traces of thymine-derivatives in the nucleotide fraction of the 1-day-blastoderm give further support for the assumption of degradation of extra-embryonal nucleic acid during the earliest development.

For the adenine nucleotides finally, the absence of details about proportions of individual adenine nucleotides, especially ADP and ATP which might have given information about energy conditions in the embryo cells, leaves little more to be stated about them here than a recognition of the fact that they are indispensable in the protein synthesis. The high proportion of ATP in the white yolk reveals a marked dissimilarity in nucleotide content between the two types of yolk, the white yolk being more in accordance with conditions in ordinary animal cells. Here should be mentioned that there exist distinct differences in amino acid content too between the white and the yellow yolk (SCHLESINGER 1951).

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Bilirubin, Alkaline Phosphatase and Transaminases in Blood and Lymph during Biliary Obstruction in the Cat

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Abstract

CARLSTEN, A., Y. EDLUND and O. THULESIUS. *Bilirubin, alkaline phosphatase and transaminases in blood and lymph during biliary obstruction in the cat.* Acta physiol. scand. 1961. *53.* 58—67. — The mechanism of hyperbilirubinemia and enzyme disturbances in acute biliary stasis have been studied experimentally in the cat. Obstruction of the common bile duct (I) and retrograde infusion of BSP and bilirubin solution (II) into the common bile duct towards the liver at pressures exceeding the maximal secretory level was performed. From these studies the following conclusions may be drawn: (1) In biliary stasis bile products simultaneously reach the blood through the hepatic vein and the liver lymph. The role of the lymphatic pathways to the accumulation of bile products seems to be minimal. (2) The rise in alkaline phosphatase activity in lymph and blood after biliary stasis is only moderate in the cat. (3) After biliary stasis there is a significant increase in transaminase activity in both blood and lymph.

Already in 1795 SAUNDERS reported the appearance of bile-tinged liquid in lymph vessels from the liver. Later studies confirmed these observations and claimed that the main mechanism responsible for the development of obstructive jaundice consisted of a primary regurgitation of bile to the liver lymph vessels from which the bile products reached the blood through the thoracic duct (MAYO and GREENE 1929, SHAFIROFF, DOUBILET and RUGGIERO 1939,

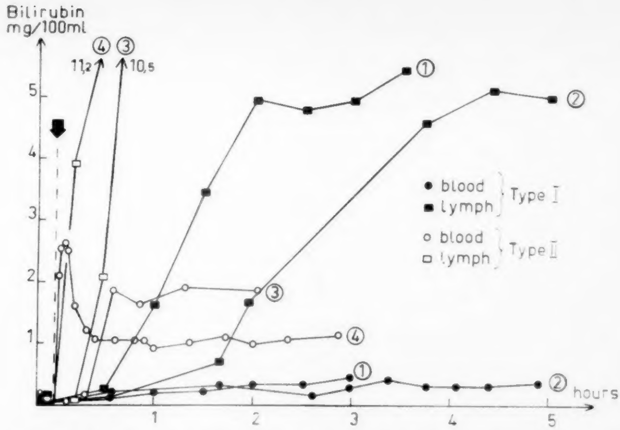

Fig. 1. Bilirubin (total) in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion of bilirubin solution (Type II). Arrow indicates onset of obstruction or infusion.

Shafiroff *et al.* 1942, 1944, Mallet-Guy *et al.* 1959). According to others bile products may reach the blood circulation either directly within the liver through radicles of the hepatic vein (Hanzon 1952, Brauer, Leong and Holloway 1954) or mainly by the way of lymphatic pathways (Bloom 1923, Gonzales-Oddone 1946). These investigators were of the opinion that bilirubin appeared first in the lymph and later in the blood. Ritchie, Grindlay and Bollman (1956) studied the mechanism with liver lymph fistulae in trained dogs. They presented evidence showing that "regurgitation" of bilirubin into the liver lymph after acute obstruction of the common bile duct was limited to the first 24 hours after onset of obstruction.

The purpose of this study was to evaluate the time relationship of the appearance of bile products in blood and lymph during the initial phase of biliary stasis in the cat. Moreover we have been interested in the pathways of some enzymes related to acute obstruction of the common bile duct.

Material and Methods

Sixteen healthy male and female cats ranging in weight from 2 to 5 kg were used in this study. The animals were anaesthetized with ether followed by intravenous chloralose — urethan (50 mg and 100 mg respectively per kg body weight). The thoracic duct was cannulated within the thorax using a plastic tube and the lymph collected in heparinized test tubes as described by Carlsten (1950). The transthoracic approach required artificial respiration which was maintained by a pump-respirator connected to a tracheal cannula.

In all experiments blood samples were obtained from the femoral vein and in some series also from the femoral artery and the hepatic vein. The blood samples were taken

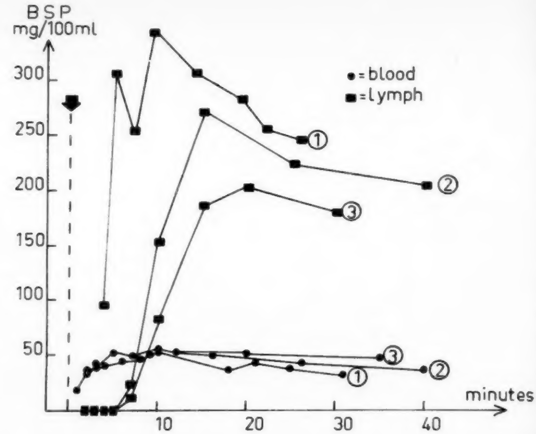

Fig. 2. Sulfobromophtalein (BSP) concentration in peripheral blood and lymph after onset of infusion.

through an indwelling polyethylene catheter. Samples from the hepatic vein were obtained by an ureteral catheter which was inserted from the right external jugular vein. The proper position of the catheter was checked by direct inspection from the abdomen where the coloured catheter easily was visible within a hepatic vein at the liver outflow. Blood and lymph samples were collected simultaneously at different intervals.

In all experiments the cystic duct of the gallbladder was tied before ligation or cannulation of the common bile duct.

Essentially 2 types of experiments were performed.

Type I. Ligation of the distal part of the common bile duct near the duodenal wall.

Type II. Cannulation of the common bile duct (after ligation of the distal part) with a plastic catheter connected to an infusion system which could be adapted to a constant hydrostatic pressure level. By this arrangement bilirubin solution (3 % "Merck", made of bile calculi from swine) or sulfobromophtalein (BSP) (5 %) could be instilled at different pressures (300—450 mm H_2O).

Chemical analyses: Direct and indirect reacting bilirubin was estimated by the method of JENDRASSIK and CLEGHORN (1936) and JENDRASSIK and GROF (1938). Alkaline phosphatase activity was determined according to the technique of BUCH and BUCH (1939), glutamic-oxaloacetic acid transaminase (GOT) and glutamic pyruvic transaminase (GTP) according to KARMEN, WROBLEWSKI and LADUE (1955). Sulfobromophtalein (BSP) concentration was determined by the method of SELIGSON, MARINO and DODSON (1957).

Results

In our first experiments blood samples were obtained from both peripheral vessles (femoral artery and vein) as well as from the hepatic vein. In experiments according to type I (ligation of the common bile duct) no significant difference in blood concentration of bilirubin and enzymes could be detected in samples from peripheral or central blood. Only slightly higher concentrations of bilirubin (Fig. 1) and BSP could be observed in samples from the hepatic vein in the infusion experiments within the first period of 30 min (type

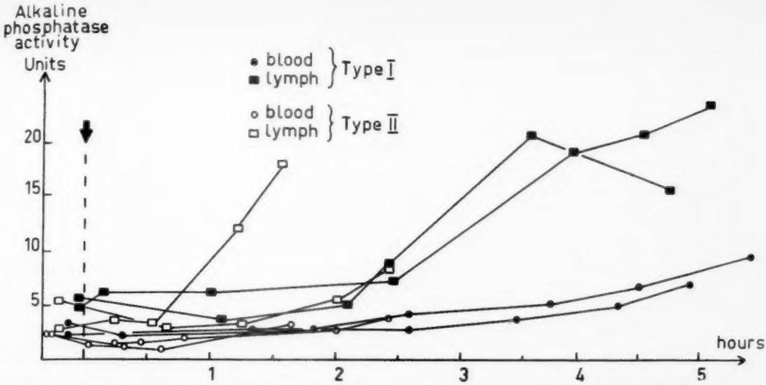

Fig. 3. Alkaline phosphatase activity in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion (Type II) of bilirubin and BSP solutions. Arrow indicates onset of obstruction or infusion.

II). Later the same concentrations were observed in peripheral and central blood.

The lymph-flow per hour from the thoracic duct ranged from 1.4—5.6 ml/kg of body weight (mean: 2.9 ml/kg). No increase of the lymph-flow values could be observed in our experiments following biliary obstruction. In some experiments according to type I the maximal biliary secretion pressure was measured. This was found to be 280—350 mm H_2O.

A. *Studies on Bilirubin Concentration*

Type I. Four experiments were performed in this group with occlusion of the common bile duct. Blood from the femoral artery and thoracic duct lymph was analysed with respect to total bilirubin. The results of two typical experiments are shown in Fig. 1. The bilirubin values showed a slight increase in blood and lymph during the first hour of biliary stasis in all 4 animals. Thereupon followed a marked increase in the bilirubin concentration of the lymph. In the blood the bilirubin level increased only slowly and reached values outranging the normal concentration (two standard deviations above the normal mean value) one hour after the onset of biliary stasis.

Type II. In this series 3 experiments were performed with infusion of bilirubin solution into the common bile duct. Two of these are shown in Fig. 1. In 2 animals the bilirubin concentration in blood (from femoral artery and hepatic vein) increased markedly already 1 minute after the infusion was started. In the third a significant rise in blood concentration could be shown to appear 35 min after the onset of infusion. The result of this experiment as well as one example with the short appearance time are plotted in Fig. 1. In

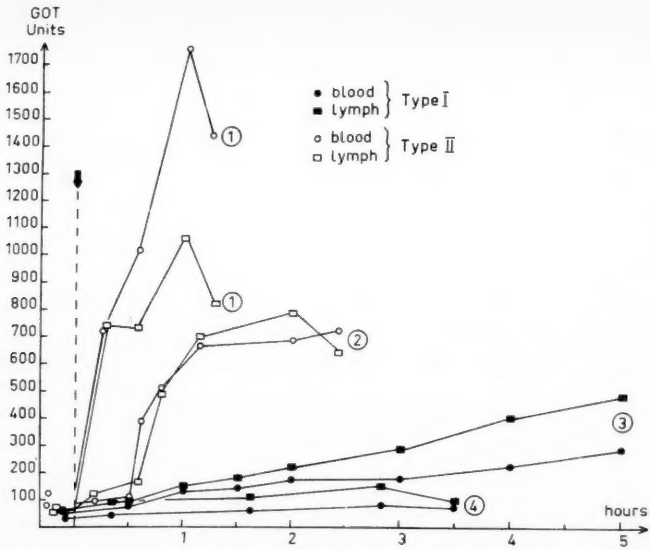

Fig. 4. GOT-activity in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion (Type II) of bilirubin (2) and BSP-solutions (1). Arrow indicates onset of obstruction or infusion.

only one experiment the lymph flow was sufficient to obtain repeated samples at short intervals. In this instance a sharp rise of the bilirubin concentration in the lymph was observed at 5 minutes after the onset of infusion. Also in these experiments lymph concentrations always exceeded blood values markedly.

B. *Infusion of BSP*

In this group 4 experiments were made with infusion of 5—12 ml of BSP-solution at a constant pressure of 450 mm H_2O (see Fig. 2). We succeded to get repeated lymph samples at short intervals within the first five minutes in two instances. In one experiment only blood from the femoral artery and hepatic vein was collected. Already the first blood samples withdrawn after 1—2 min contained measurable amounts of BSP. In the lymph BSP could be detected after 4—7 min. After the first "peak values" in blood and lymph the concentration in the subsequent periods declined, the lymph concentration always remaining highest.

C. *Studies on Enzyme Activity*

Alkaline phosphatase: In experiments with simple ligation of the common bile duct (type I) the alkaline phosphatase activity shows an even more delayed increase in blood and lymph compared with the bilirubin values. A significant

Table I. Bilirubin, alkaline phosphatase, GOT and GPT in blood and lymph in 10 animals before biliary obstruction (mean values $\pm$ standard deviation)

	Bilirubin (mg/100 ml)		Alkaline Phosphatase (units)	GOT (units)	GPT (units)
	Total	Direct			
Blood......	0.12 ± 0.04	0.07 ± 0.02	2.8 ± 1.5	65 ± 21	32 ± 15
Lymph	0.12 ± 0.07	0.09 ± 0.07	2.9 ± 1.6	63 ± 26	24 ± 7

rise of phosphatase activity in lymph appearing after 3 1/2 hours with lower values in the blood. In experiments with infusion of bilirubin solution or BSP (type II) the rise of alkaline phosphatase activity appeared somewhat earlier in the lymph in one experiment (see Fig. 3).

GOT: In experiments performed according to type I a slight rise in GOT activity could be observed in lymph and blood during 5 hours after biliary obstruction. Intrabiliary infusion with BSP or bilirubin was after a short interval followed by a markedly increased GOT activity (within 35 min (Fig. 4)). When sufficient amounts of lymph could be obtained also GPT-activity was determined. In these experiments activity curves followed the same pattern as GOT only at slightly lower levels.

Discussion

Bilirubin is transported in the blood to the liver which excretes the pigment in bile. Passage through the liver produces some change in the pigment which is detectable by the direct van den Bergh reaction. The difference between direct and indirect-reacting bilirubin is due to the conjugation of lipid-soluble bilirubin (indirect-reacting) with glucuronic acid to water-soluble bilirubin-diglucuronide (direct-reacting). Both direct and indirect reacting bilirubin were found to be normal constituents of thoracic duct lymph (see Table I), 30 % of which is derived from the liver (Morris 1956, Ritchie-Grindlay and Bollman 1959). The water soluble bilirubin-glucuronide probably reaches the lymph in the portal canals by diffusion from bile ducts which, according to Bollman (1951), are richly sourrounded by anastomosing networks of lymph vessels, moreover bilirubin-glucuronide and the bilirubin-albumin complex can pass to the lymph via the pericapillary spaces (Fig. 5).

Under conditions of biliary obstruction there is a successive rise of biliary pressure which gradually interferes with the passage of bile constituents from blood to the hepatic cell ("retention" of indirect reacting bilirubin) and from the cell to bile canaliculi. In spite of the increased pressure gradient there is still a limited transfer of bilirubin into the hepatic cells and to adjacent bile capillaries and back to the pericapillary space and sinusoids *e. g.* to lymph and blood ("regurgitation" of direct reacting bilirubin) (Brauer *et al.* 1954).

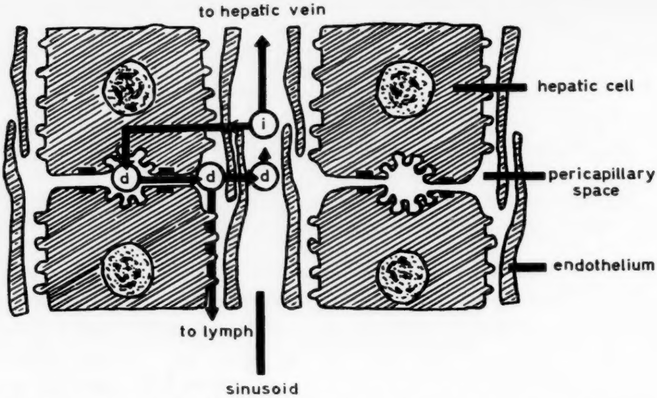

Fig. 5. Schematic drawing of the functional unit of a liver lobule. Arrows indicate pathways of bilirubin under normal conditions and during biliary stasis. (i = indirect reacting bilirubin, d = direct reacting bilirubin.) For further details see text.

Our studies on bilirubin concentration in blood and lymph after ligation of the common bile duct (type I) are in accordance with previous investigations (with higher concentrations of bilirubin in lymph). A significant elevation of the bilirubin concentration above normal values seems to be brought about earlier in the lymph. This has led to the concept of the initial transfer of bile constituents to the lymph thus reaching the blood stream through the thoracic duct followed later by direct passage to the blood. These studies did, however, not take into consideration the quantitative relationship of the blood and lymph compartments. Assuming that both the thoracic duct lymph and the circulating blood volume received an equal amount of bilirubin at the same time after the onset of biliary obstruction it is obvious that a significant rise in concentration can be traced earlier in the lymph.

From the above mentioned anatomical considerations it is reasonable to assume that bile products regurgitate simultaneously into blood and lymph (via sinusoids *cf.* pericapillary space). Our infusion studies present strong evidence in favour of such a mechanism. In fact, infusion of BSP resulted in a shorter appearance time of this test substance in blood from the femoral artery than in thoracic duct lymph. The time necessary for transportation of the indicator from the liver to the thoracic duct can be considered responsible for this short delay (4—5 min). It is therefore reasonable to assume that bile substances reach liver lymph and hepatic vein blood virtually simultaneously. The conclusions may be open to some criticism because they apply only to BSP. Nevertheless the great similarity between BSP and bilirubin has been stressed by many investigators who claim an active transport mechanism connected with a proc-

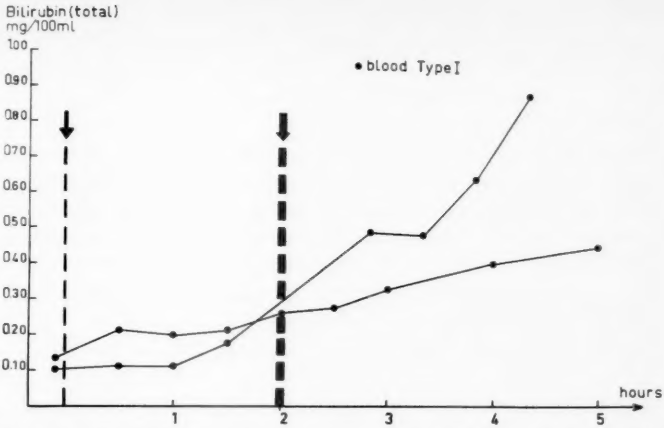

Fig. 6. Bilirubin concentration in peripheral blood (femoral vein) after obstruction of common bile duct (thin arrow). Thick arrow and dotted line (at 2 hours) indicate cannulation of thoracic duct with removal of lymph.

ess of conjugation (Wheeler, Meltzer and Bradley 1960), in some way similar to the excretory pattern of bilirubin.

The related experiments do not disclose to what extent the lymphatic system quantitatively contributes to the accumulation of bile products in the blood. We presume, however, that these are predominantly discharged from the hepato-biliary system directly into the blood and not through lymphatic pathways. Evidence for this assumption can be found in 2 experiments in which the thoracic duct was cannulated 1 hour after ligation of the common bile duct (Fig. 6). Under these conditions the initial rise of bilirubin concentration in blood with intact lymph-flow continued with the same slope after delayed cannulation of the thoracic duct and removal of lymph from the blood circulation.

The mechanism responsible for hyperphosphatasemia in biliary stasis is not fully understood. It is generally suggested that the alkaline phosphatases produced in various tissues are excreted by liver in the bile (Freeman and Chen 1938). The considerations led to the concept of phosphatase "regurgitation" analogous to the mechanism leading to hyperbilirubinemia (Freeman, Chen and Ivy 1938, Edlund 1952). If this was the case then the concentration pattern of phosphatases in blood and lymph in experimental biliary stasis should be similar to that of bilirubin. This is, however, not entirely the case in our experiments in which minor differences could be observed with a delayed rise in phosphatase activity, compared with bilirubin concentration values. Cantarow, Stewart and McCool (1936) and Flood, Gutman and Gutman (1937) found only a slight rise in the phosphatase activity after chronic obstruc-

tion of the common bile duct in the cat. They considered this rise to be negligible in comparison with the marked elevations regularly observed following obstruction of the common bile duct in the dog and in man. These authors claimed that this difference was due to excretion of serum phosphatases through the kidney. They found that the urine of the cat, unlike that of man and the dog, normally contained considerable amounts of alkaline phosphatases. It is somewhat surprising to find such a moderate increase of the phosphatase activity after biliary obstruction and on infusion with BSP or bilirubin solution in our experiments. To some extent this may be explained by renal excretion of phosphatase from the blood. On the other hand not even the lymph values are very impressive and these are not immediately influenced by renal activity. The actual experiments are, however, limited to observations within 5 hours.

Biliary obstruction could be shown to be followed by steadily increasing GOT activity in blood and lymph. In the infusion experiments an early and even pronounced elevation in GOT-activity could be shown to appear in both lymph and blood. CHINSKY, SHMAGRANOFF and SHERRY (1957), CHINSKY and SHERRY (1957) and DUNN, MARTIAS and REISSMANN (1958) among others have shown that GOT is excreted in the bile in man and in the dog but there is no renal excretion. The very marked rise in GOT activity in the infusion experiments can hardly be explained alone by regurgitation of GOT from the biliary tree. The liver cells with their high content of transaminases must have contributed to this mechanism, the only possible explanation being liver cell damage (BRAUN, PAPP and HORVÁTH 1959), which in our experiments in some way must be related to a toxic effect of the highly concentrated dye (5 %) or bilirubin (3 %). In 3 control experiments with infusion of saline (0.9 %) and Dextrane (Macrodex) into the common bile duct no sharp rise in transaminase activity could be detected. Interference of the high BSP and bilirubin concentrations on the method of GOT determination could be excluded in various controls.

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The Effect of Chlorpromazine on the Glucose Metabolism in Different Parts of the Goat Brain

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Abstract

Larsson, S. *The effect of chlorpromazine on the glucose metabolism in different parts of the goat brain.* Acta physiol. scand. 1961. *53.* 68—74. — Small doses of chlorpromazine were given parenterally to adult goats. Different parts of the hypothalamus and of the cerebral and cerebrellar cortici as well as of the posterior and anterior pituitary were then incubated with generally labelled ^{14}C glucose. It was found that the respiratory exchange as well as the ^{14}C lactic acid formation was depressed by the drug in the hypothalamus and in the posterior pituitary. The formation of amino acids from exogenous glucose was also inhibited in the same areas after administration of chlorpromazine. The fate of ^{14}C-labelled glucose was essentially unaffected by chlorpromazine in the cerebral and cerebellar cortici.

In series of experiments the fate of ^{14}C-labelled glucose in different parts of the brain and in the pituitary has been studied with the use of a technique for quantitative paper radio-chromatography (Beloff-Chain *et al.* 1959, Chain, Larsson and Pocchiari 1960, Andersson, Larsson and Pocchiari 1961 and Larsson 1961). Thus, it has been found that the formation of amino acids from exogenous ^{14}C glucose is comparatively high in the brain and in the pituitary compared to other tissues. In addition, the different parts of the brain have different metabolic patterns, both quantitatively and qualitatively.

As chlorpromazine has been found to accumulate in certain parts of the brain, namley the hypothalamic area (Wase, Christensen and Polley 1956), the present study was undertaken to see if this substance also caused quantitative or qualitative changes in the metabolic pattern in different parts of the brain.

Material and Methods

Generally labelled ^{14}C glucose was obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give a specific activity of 20 μC per mg.

The composition of the incubation medium was as follows: NaCl 0.098 M, KCl 0.027 M, $MgSO_4$ 0.0012 M, KH_2PO_4 0.0004 M, Na_2HPO_4 0.0175 M, and radioactive glucose 0.0056 M—3.6 mC per mM at pH 7.3.

Adult female goats were used for the experiments. The animals were injected with 6.0—7.5 mg Hibernal, LEO (chlorpromazine) intravenously. The dose corresponded roughly to 0.25 mg per kg body weight.

About 15 min after the injection the animals were killed by decapitation. The injected drug caused a mild to moderate sedation.

The animals were killed by decapitation after which the brain and the pituitary were dissected out as quickly as possible. The following tissue samples were taken for incubation:

1. Periventricular hypothalamic tissue (PV).
2. Ventromedial hypothalamic tissue (VM).
3. Tissue from the cerebral cortex.
4. Tissue from the cerebellar cortex, taken medially along the dorsal curvature.
5. Tissue from the posterior pituitary (PP).
6. Tissue from the anterior pituitary (AP).

The exact localisation of the samples, except for no. 4, has been given elsewhere (Andersson *et al.* 1961, Larsson 1961).

The incubation conditions were the same as previously reported (Andersson *et al.* 1961). Thus, the tissue was incubated for 1 hour at 37° C, the gas phase being pure oxygen. After incubation the medium was immediately transferred to paper for chromatography and the tissue treated as described by Chain *et al.* (1960). The solvents for the chromatography were also the same as those used by the previous authors. The chromatograms were scanned quantitatively by a transistorised version (Larsson and Ström) of the automatic device described by Frank *et al.* (1959).

The formation of $^{14}CO_2$ by the tissue samples was determined as described by Villee and Hastings (1949). The insoluble residue after the extraction of the tissue was not studied.

For the statistical analyses the *t*-test was employed. The following symbols were used:
*** = differences highly statistically significant
** = differences statistically significant (at the 5 % level or better)
* = differences almost statistically significant (at the 5 % level).

Results

Table I gives the values for oxygen consumption, $^{14}CO_2$- and ^{14}C lactic acid formation in the different parts of the brain and the pituitary, with and without previous administration of chlorpromazine. From the table it is

Table I. The effect of chlorpromazine on the conversion of ¹⁴-labelled glucose into $^{14}CO_2$ and ^{14}C lactic acid, and the oxygen consumption in different parts of the brain and the pituitary

Results expressed as μg of glucose converted based on the fraction of total radioactivity of glucose incorporated per 25 mg of tissue (wet wt.) after 1 h incubation at 37°C in O_2 in 0.5 ml of medium. Oxygen consumption expressed in μl per wt. unit. Glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values $\pm$ s. e. m.

Chlorprom.		PV	VM	Cort. cerebr.	Cort. cerebell.	PP	AP
O_2	0[1]	38.7 ± 3.1 (14) ***	34.0 ± 3.3 (10) ***	36.2 ± 3.7 (6)	35.2 ± 4.5 (3)	30.5 ± 3.2 (10) ***	26.1 ± 2.6 (9)
	+	14.0 ± 0.6 (5)	14.8 ± 0.6 (5)	30.7 ± 2.7 (5)	28.4 ± 3.2 (3)	15.2 ± 2 0 (5)	18.7 ± 2.5 (4)
$^{14}CO_2$	0[1]	11.9 ± 1.0 (14) ***	10.8 ± 1.9 (10) ***	10.5 ± 1.4 (6)	10.0 ± 2.8 (3)	8.7 ± 1.6 (10) **	2.3 ± 0.3 (8)
	+	5.0 ± 0.6 (5)	5.0 ± 0.7 (5)	10.6 ± 0.7 (5)	9.7 ± 0.5 (3)	5.3 ± 0.4 (4)	1.3 ± 0.4 (3)
^{14}C-lact. acid	0[1]	102.3 ± 7.2 (13) ***	89.8 ± 4.3 (10) *	103.3 ± 9.7 (6)	100.1 ± 10.0 (3)	72.3 ± 5.1 (10) ***	30.3 ± 1.3 (8) *
	+	60.3 ± 2.4 (5)	67.2 ± 2.0 (5)	81.5 ± 4.9 (5)	84.4 ± 6.3 (3)	42.8 ± 4.0 (5)	17.9 ± 2.5 (4)

[1] values derived from ANDERSSON et al. (1961), except from *cerebrum* (LARSSON 1961) and from cerebellum (present study)
Abbreviations of tissue samples — see Materials and Methods
Number of experiments within brackets

evident that the drug administration significantly depressed the oxygen consumption, $^{14}CO_2$- and ^{14}C lactic acid production in the periventricular and ventromedial hypothalamus as well as in the posterior pituitary. In the other parts studied there were essentially no changes in these parameters whether chlorpromazine was administered or not.

Table II shows the formation of ^{14}C-labelled amino acids in the brain and in the pituitary from the ^{14}C glucose in the medium, and how chlorpromazine adminstration influenced this pattern. It was found that the drug decreased the formation of all amino acids, except alanine, from the exogenous glucose in the periventricular tissue. In the ventromedial hypothalamic samples the inhibition was less pronounced. In the posterior pituitary, glutamic acid formation, in particular, was decreased by chlorpromazine. This was also true for the samples from the anterior pituitary. Further, in this part the drug

Table II. The effect of chlorpromazine on the conversion of ^{14}C-labelled glucose into various amino acids in different parts of the brain and the pituitary

Results expressed as μg of glucose converted based on the fraction of total radioactivity of glucose incorporated per 25 mg of tissue (wet wt.) after 1 h incubation at 37°C in O_2 in 0.5 ml of medium. Glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values ± s. e. m.

Tissue	Chlor-prom.	Glutamic acid	GABA	Alanine	Aspartic acid	Glutamine	Total
PV	[1]0	6.0 ± 0.7 (14) ***	3.5 ± 0.3 (14) **	1.4 ± 0.1 (14)	0.9 ± 0.1 (14) **	1.2 ± 0.3 (14) ***	13.0 (14)
	+	2.7 ± 0.2 (5)	2.0 ± 0.2 (5)	1.4 ± 0.4 (5)	0.2 ± 0.02 (5)	0.3 ± 0.01 (5)	6.6 (5)
VM	[1]0	4.4 ± 0.6 (10) **	2.6 ± 0.5 (10)	1.3 ± 0.3 (10)	0.5 ± 0.1 (10) *	1.1 ± 0.2 (10) ***	9.9 (10)
	+	2.5 ± 0.2 (5)	1.8 ± 0.3 (5)	1.4 ± 0.4 (5)	0.2 ± 0.1 (5)	0.3 ± 0.01 (5)	6.2 (5)
Cortex cerebr.	[2]0	6.4 ± 0.8 (6)	1.3 ± 0.3 (6)	1.4 ± 0.5 (6)	0.8 ± 0.2 (6)	1.5 ± 0.4 (6) *	11.4 (6)
	+	5.5 ± 0.4 (5)	1.6 ± 0.2 (5)	1.3 ± 0.05 (5)	0.7 ± 0.02 (5)	0.7 ± 0.02 (5)	9.8 (5)
Cortex cerebell.	0	5.0 ± 0.6 (3)	1.1 ± 0.3 (3)	1.5 ± 0.4 (3)	0.7 ± 0.3 (3)	0.9 ± 0.5 (3)	9.2 (3)
	+	4.6 ± 0.4 (3)	1.4 ± 0.2 (3)	1.7 ± 0.1 (3)	0.8 ± 0.1 (3)	0.2 ± 0.02 (3)	8.7 (3)
PP	[1]0	4.1 ± 0.5 (10) ***	1.0 ± 0.03 (10)	0.7 ± 0.2 (10)	0.8 ± 0.1 (10) **	0.7 ± 0.1 (10)	7.3 (10)
	+	1.8 ± 0.3 (5)	1.2 ± 0.1 (5)	0.9 ± 0.2 (5)	0.2 ± 0 (5)	0.8 ± 0.1 (5)	4.9 (5)
AP	[1]0	0.7 ± 0.1 (8) *		0.9 ± 0.1 (8) *	0.4 ± 0.05 (8) *	0.9 ± 0.2 (8) *	2.9 (8)
	+	0.2 ± 0.1 (4)	0.2 ± 0.1 (4)	0.3 ± 0.1 (4)	0.1 ± 0.02 (4)	0.4 ± 0.01 (4)	1.2 (4)

[1] values derived from Andersson et al. (1961)
[2] values derived from Larsson (1961)
GABA = γ-aminobutyric acid
Abbreviations of tissue samples — see Materials and Methods
Number of experiments within brackets

caused the appearance of small amounts of γ-aminobutyric acid. In the samples from the cerebrum and the cerebellum essentially no changes were observed after chlorpromazine injection.

Discussion

When ^{35}S-chlorpromazine was administered parenterally into rats, accumulation of radioactivity in the hypothalamic area of the brain was encountered (WASE *et al.* 1956). In the same study it was also found that the drug caused a marked decrease in the phospholipid turnover of the hypothalamus. In the present study, with a different approach, it was found that the administration of chlorpromazine in relatively small, therapeutic doses, caused a decrease in the respiratory exchange of the hypothalamic tissue samples as well as of the posterior pituitary. The cortical samples from the cerebrum and the cerebellum were unaffected by the drug in this respect. In the present experiments the metabolic response of chlorpromazine was studied in "resting" tissue. However, MCILWAIN and GREENGARD (1957) found that chlorpromazine was a potent inhibitor of the metabolic response to electrical pulses in isolated cerebral tissue (cortex) in concentrations of 5 to 10 μM. In the present study on cerebral and cerebellar cortici, even if the oxygen consumption had a tendency to be less after chlorpromazine administration, the differences between normal and injected animals were not significant. ERNSTING *et al.* (1960) studying certain metabolic trends of psychotropic drugs, found that chlorpromazine depressed oxygen consumption in brain slices. According to their description of the experimental procedures it seemed that the authors did not study the tissue in the di- and mensencephalon. WASE *et al.* (1956) found that the accumulation of ^{35}S from ^{35}S chlorpromazine after repeated injections almost exclusively was encountered in the lipid fraction. This was true for the brain as well as for the plasma and for the liver. Further, the most pronounced accumulation of the drug in the brain was in the hypothalamic area as was the decrease in phospholipid turnover due to the drug. As mentioned before, the present results indicated the most marked and significant depression of the respiratory exchange as well as of the ^{14}C lactic acid and ^{14}C amino acid formation due to the drug in the hypothalamus and in the posterior pituitary. Accumulating results have shown that when in normal animals different parts of the brain were incubated with ^{14}C glucose or ^{14}C fructose appreciable amounts of ^{14}C-labelled amino acids were found in the tissue extracts (CHAIN *et al.* 1960, ANDERSSON *et al.* 1961, and LARSSON 1961). However, when regarding the different parts of the brain they will show amino acid formation pattern which is rather characteristic for the part *per se*, both quantitatively and qualitatively (previous references). Even the two parts of the pituitary will differ in this respect (ANDERSSON *et al* 1961, and LARSSON 1961). Generally one can say that this pattern follows that of phylogenetical origin. However, it has also been shown that merely the

state of hunger can cause marked metabolic differences even within areas of the same phylogenetical origin (FORSSBERG and LARSSON 1954, 1955). In the present study, further, it was found that the administration of chlorpromazine in general did not affect the fate of exogenous ^{14}C glucose in the cerebral and cerebellar slices but did so in the periventricular and ventromedial hypothalamic samples as well as in the posterior pituitary, and partly also in the anterior pituitary. As previously discussed, *in vitro*, chlorpromazine caused an inhibition of the oxygen consumption (ERNSTING *et al.* 1960). In these experiments, however, the substance was present in comparatively high concentrations and incubated with the tissue. According to ERNSTING *et al.* (1960) the action of chlorpromazine should be dual, one mode of action being active on certain phases of the respiratory chain, and one on the membrane permeability. According to the authors the latter effect should be the most predominant. In the present experiments it was found that in the periventricular tissue the formation of all amino acids analysed here but alanine was depressed by chlorpromazine. In the ventromedial hypothalamic tissue this effect was less pronounced, but still significant. Partly, this was also true for the posterior pituitary. Thus, if one forms the ratio between the formation of glutamic acid and of γ-aminobutyric acid in the different parts of the hypothalamus and in the posterior pituitary one will find that the administration of chlorpromazine will make the ratio to approach one, from normally being well above this figure.

In this connection it is of interest to note that the posterior pituitary behaved like the hypothalamic samples towards the administration of chlorpromazine. "Normally" the posterior pituitary has many similarities with the hypothalamic parts regarding the fate of ^{14}C glucose in the incubation medium (ANDERSSON *et al.* 1961, and LARSSON 1961). Thus, in this respect also, there is a good agreement with what is known about the physiological and histological properties concerning this part in relation to certain areas of the hypothalamus. It was therefore not surprising that chlorpromazine was found to affect the metabolism of the posterior pituitary in a similar way as in the hypothalamus.

Partly in contrary to the findings by ERNSTING *et al.* (1960) the present study showed that the rate of conversion of radioactive glucose into various amino acid was depressed due to chlorpromazine in the hypothalamus and in the pituitary. However, they measured the total amounts of the amino acids while in the present experiments the rate of conversion from exogenous glucose has been measured.

It thus seems that at a higher central level chlorpromazine will inhibit certain aspects of the metabolism of the hypothalamus, the posterior pituitary and partly the anterior pituitary, in which latter part γ-aminobutyric acid was found. The present study is taken as a support for the theory that the action of a neuropharmaca will substanciate in biochemical changes specific to the part of the brain where the drug acts.

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The Effect of Deuterium Oxide on the Mechanical Properties of Muscle

By

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Abstract

Svensmark, O. *The effect of deuterium oxide on the mechanical properties of muscle.* Acta physiol. scand. 1961. *53.* 75—84. — The effect of deuterium oxide on the isometric contraction of frog's muscle was investigated. In 99.8 per cent deuterium oxide the twitch force was reduced to 2—70 per cent of the force in aqueous Ringer, the rate of force development to 12—30 per cent, the tetanic force to 61—96 per cent while the latency of force development was approximately doubled. The total duration of the twitch was unchanged. The duration of the plateau of maximum intensity of active state remained unaltered by deuterium while its decline was enhanced. The conduction time of the action potential was not affected by deuterium but the reduction in frequency at which a complete tetanus could be maintained indicated an effect on the excitatory processes. It remained undecided whether the reduction in force is due to an effect of deuterium on the contractile elements or on the excitation-contraction coupling.

It is well known that deuterium inhibits many enzymatic reactions (Katz 1960, Kritchevsky 1960). An enzyme associated with muscular activity, adenosine triphosphatase was inhibited by 50 per cent in 90 per cent deuterium oxide as is seen from the appendix p. 83. It seemed of interest to investigate whether this inhibition is reflected in the mechanical response of skeletal muscle. This report deals with the effect of deuterium oxide on the isometric twitch force, the rate of force development, the latency of force development, the duration of twitch, the tetanic force and the duration of the active state of frog's muscle.

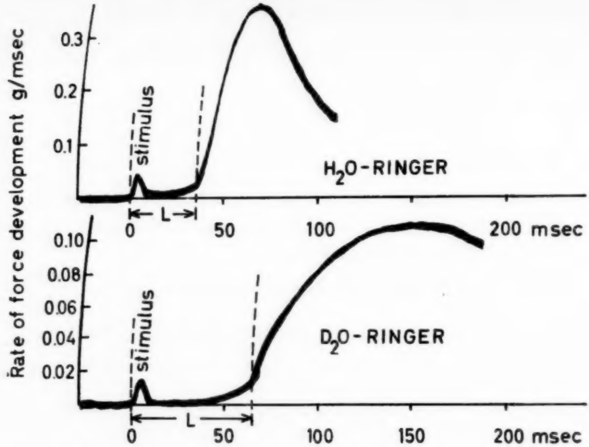

Fig. 1. Definition of the latency of maximum acceleration as the time interval L between onset of stimulation and maximum acceleration of the force development. Record of the time course of the rate of force development in isometric twitches at 2° C. Upper curve: in H_2O-Ringer. Lower curve: in 99.8 per cent D_2O-Ringer. (M. semitendinosus; weight 26 mg; length 18 mm; stretched 10 per cent above equilibrium length.)

Methods

The experiments were made on the semitendinosus muscle. The frogs (*Rana esculenta*) were curarized (50 μg d-tubocurarine per g frog) and one belly of the muscle (15—40 mg) with a maximum diameter of 0.5 to 1 mm was placed in a thermostated chamber at 2° C and bathed in a Ringer solution with either water or 99.8 per cent deuterium oxide as solvent. The composition of the Ringer was: NaCl 115 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, sodium phosphate buffer 2.5 mM, pH 7.3 and d-tubocurarine 50 μg per ml.

The muscle was stimulated from end to end or, to reduce the conduction time, 'transversely' by a multielectrode assembly extending over the entire length. Two assemblies each consisting of five platinum electrodes of alternate polarity were placed on each side at a distance of 4 mm from the axis of the muscle. The stimuli were supramaximal rectangular pulses 1.5 msec in duration. Before each experiment the muscle was stimulated with single stimuli at intervals of one minute for a period of one hour; only muscles with constant twitch force within this period were used. During the experiments the same rate of stimulation was maintained. In addition, tetanic contractions were evoked at intervals of ten minutes by trains of 15 stimuli per sec and a train duration of 1—2 sec. At this low frequency of stimulation the tetanus was almost complete and maximum force was attained, the change in force per stimulus being less than one per cent. The low frequency was chosen since at higher frequencies the tetanic force decreased in deuterium oxide.

The isometric force was recorded by a Brush ink writer *via* an electromechanical transducer (RCA 5734). The sensitivity of the recording system allowed a change of 100 mg to be determined. The response of the recorder to a stepwise increase in force had a rise time of 10 msec for a full scale deflection, and at the low temperature used

Table I. Rate of deuterium exchange with muscle hydrogen at 0° C. A semitendinosus muscle (19.5 mg) was incubated with 50 per cent D_2O-Ringer for 1 hour at 20° C and immersed in 2 ml water at 0° C. The deuterium concentration was determined in the outer fluid at various time intervals.

Time of exchange minutes	Concentration of deuterium oxide in the outer fluid (per cent)
0.8	0.122
2.1	0.352
5.2	0.447
10	0.482
20	0.485
40	0.493

(2° C) the time course of force development was recorded without essential distortion. The resting length of the muscle corresponded to a load of 2 per cent of the tetanic force. To determine the rate of force development with greater accuracy than obtainable from force recordings, the electronically differentiated signal from the transducer was recorded simultaneously. The open loop gain of the differentiator amplifier was one hundred, and the time constant of the differentiating network was 33 msec. To obtain a well defined determination of the latency of the mechanical response the time interval was measured between onset of stimulation and the time at which the slope of the differentiated record of the force development was maximum (L in Fig. 1). This interval corresponds to the latency of maximum acceleration of the change of force and is in the following denoted as 'latency'. To obtain a sufficient measuring accuracy the amplification was adjusted to about identical maximum deflections before and after exposure to D_2O. Application of D_2O resulted in the same relative increase in latencies of the inflection points, of half peak and peak rate of force development. The latency of maximum acceleration was about twice the latency estimated from the beginning of force development.

The active state is defined as the load which the contractile substance can just carry without lengthening (Hill 1949). The duration of the 'plateau' of the active state and its decline during a twitch were determined by the quick release method described by Ritchie (1954). The release (10 per cent of the muscle length) was obtained by electromagnetic displacement of a stiff steel wire to which the muscle was attached. The displacement mechanism was released by the stimulus *via* a time-delay circuit.

Determination of the exchange of deuterium oxide with muscle water[1]*:* Semitendinosus muscles of about 20 mg were incubated for one hour with 50 per cent deuterium oxide Ringer at 20° C. After removal of adhering fluid with filter paper the muscle was immersed in 2 ml water at 0° C. After efficient stirring 15 μl samples were cryosublimated and the deuterium content in the cryosublimate was determined in a density gradient tube. (For details of the technique see: Hvidt *et al.* 1954, Krause and Linderstrøm-Lang 1955.)

[1] These experiments were carried out in collaboration with the late professor K. Linderstrøm-Lang, The Carlsberg Laboratory, Copenhagen.

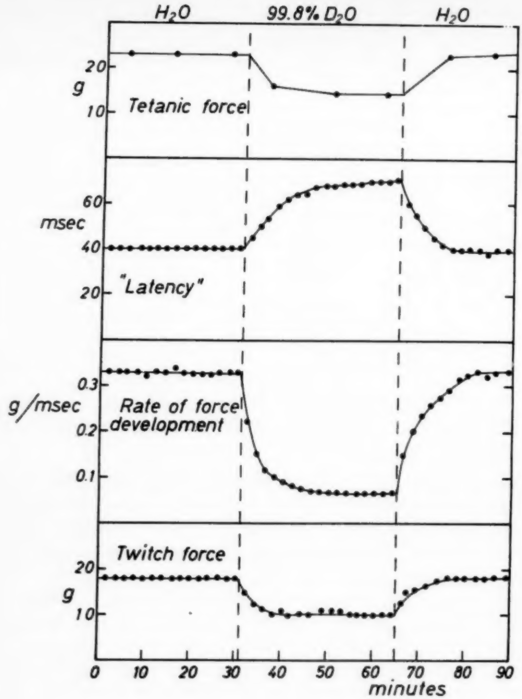

Fig. 2. Reversible effect of deuterium oxide on the twitch force, rate of force development, 'latency' (latency of maximum acceleration) and tetanic force. (M. semitendinosus; weight 28 mg; length 18 mm; stretched 10 per cent above equilibrium length.)

Results

1. *Rate of deuterium exchange with muscle hydrogen at 0° C:* An evaluation of the effects of concentrated deuterium on muscle contraction requires that the rate of exchange between deuterium and hydrogen in the muscle is known. To determine this rate the muscle was loaded with deuterium oxide and the rate of back-exchange with water was determined. In the experiment represented in Table I the total amount of deuterium released from the loaded muscle was 15 per cent higher than could be accounted for from a calculation of the water content and the exchangeable protein hydrogen of the muscle. This may possibly be due to deuterium adhering to the surface of the muscle, to a slight swelling or to selective uptake of deuterium at certain sites. Independent of this uncertainty it was, however, evident that about 98 per cent of the deuterium appeared during the first 10 min and that the exchange was nearly complete after 40 min. Although the exchange of muscle water and of easily exchangeable hydrogen occurred with half times of less than 2 min, the effects of deuterium on the mechanical response were followed for 1—2 hours to ascertain that also the exchange of more slowly exchangeable hydrogen was completed.

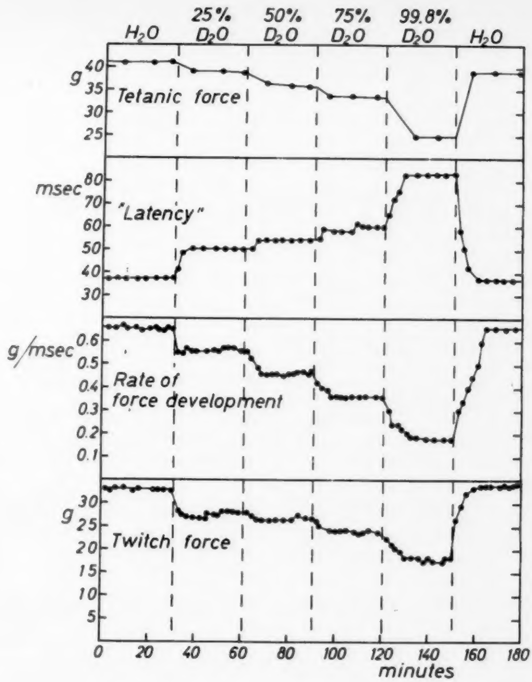

Fig. 3. Effect of different concentrations of deuterium oxide on the twitch force, rate of force development, 'latency' (latency of maximum acceleration) and tetanic force. (M. semitendinosus; weight 30 mg; length 19 mm; stretched 10 per cent above equilibrium length.)

2. *Mechanical response:* In 99.8 per cent D_2O-Ringer the twitch force, the rate of force development and the tetanic force were significantly decreased while the 'latency' increased. These changes took place in the course of the first five to fifteen minutes after replacement of H_2O-Ringer with D_2O-Ringer. The values attained after this initial period remained constant for 3—6 hours as long as D_2O was present. Upon exchange of D_2O-Ringer with H_2O-Ringer the effects of deuterium oxide disappeared within 5—15 min (Fig. 2). The total duration of the twitch was not affected by deuterium.

The changes depended on the concentration of D_2O. The rate of force development decreased nearly proportional to the concentration of D_2O whereas the effect on the twitch force, the 'latency' and the tetanic force was relatively larger at 99.8 per cent than at lower concentrations of D_2O (Fig. 3).

The twitch force averaged 16 ± 1 g (number of experiments: 30) in H_2O-Ringer. In 99.8 per cent D_2O-Ringer it decreased to 70 per cent or less in all muscles investigated; in seven muscles the twitch force was less than 15 per cent of the force in H_2O-Ringer. The rate of force development decreased from an average of 0.7 g/msec to 0.03—0.2 g/msec, i. e. by 3 to 8 times. The 'latency',

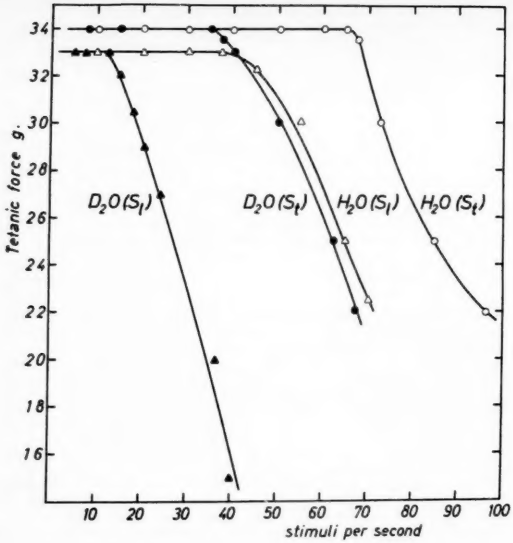

Fig. 4. The decline of tetanic orce as a function of stimulus frequency in H_2O-Ringer and D_2O-Ringer with end-to-end (S_l) and transverse (S_t) stimulation. (M. semitendinosus; weight 30 mg; length 17 mm; stretched 10 per cent above equilibrium length.)

on the other hand, increased from 30—40 msec in H_2O-Ringer to 60—80 msec in D_2O-Ringer. The tetanic force averaged 24 $\pm$ 3 g in H_2O-Ringer (30 exp.); it decreased less than the twitch force (to 61—96 per cent of the value in H_2O-Ringer). Moreover, in D_2O-Ringer the tetanic force decreased when the stimulation frequency exceeded 12—20 per second whereas it remained independent of frequency up to 40 per second in H_2O-Ringer (fig. 4).

To investigate whether the effect on twitch force, rate of force development and latency were caused by a decreased rate of propagation of the action potential along the muscle fibres, the muscle was stimulated alternately by transverse and by end-to-end stimulation. Both in H_2O- and D_2O-Ringer the 'latency' was then reduced to one half with transverse stimulation as compared with end-to-end stimulation, but twitch force, rate of force development and tetanic force remained unchanged within the accuracy of recording. In D_2O-Ringer the frequency of stimulation above which the tetanic force declined was 15—40 per second when stimulating through the multielectrode assembly as compared with 12—20 per second with end-to-end stimulation. The corresponding figures for H_2O-Ringer were 70 with the multielectrode and 40 with end-to-end stimulation (Fig. 4).

3. *Active state:* The active state curve was determined by introducing sudden releases at varying time intervals after the stimulation. In the series of twitches thus obtained the maximum force development was delayed by up to 400 msec and the peaks of these twitches represent points on the active state curve. The

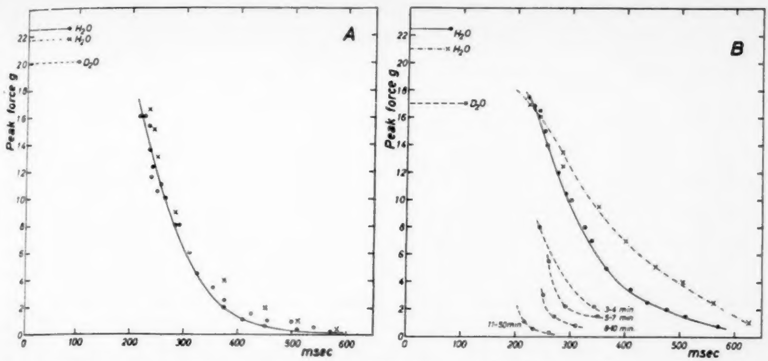

Fig. 5. 'Plateau' and decline of active state in H_2O-Ringer (●), 99.8 per cent D_2O-Ringer (○) and in H_2O-Ringer after removal of the D_2O-Ringer (×). The durations of the 'plateau' were determined after more than 30 minutes equilibration with H_2O- or D_2O-Ringer.

A: An example of the experiments in which the twitch force was reduced by D_2O to 50—70 per cent of the force in H_2O-Ringer. (M. semitendinosus; weight 24 mg; length 17 mm; stretched 10 per cent above equilibrium length.)

B: An example of the experiments in which the twitch force was reduced by D_2O to 6—20 per cent of the force in H_2O-Ringer. The decline of the active state was determined at different times (3—50 minutes) after replacement of H_2O-Ringer with D_2O-Ringer. (M. semitendinosus; weight 31 mg; length 19 mm; stretched 10 per cent above equilibrium length.)

duration of the 'plateau' of maximum intensity of active state was 30—40 msec for a single stimulus and was determined as the time interval from the last stimulus in a tetanus to the time at which the force began to fall. In D_2O-Ringer the duration of this 'plateau' of maximum intensity of the active state was prolonged by 30—40 msec, possibly due to the increase in the latent period which was of the same order.

As to the decline of the active state eleven experiments fell into two groups:

A: In 5 experiments in which the twitch force decreased in D_2O-Ringer to 70 per cent of the force in H_2O-Ringer the descending phase of the active state curves in D_2O-Ringer coincided with the lower parts of those in H_2O-Ringer (Fig. 5 A).

B: In 6 experiments in which the twitch force was reduced to less than 20 per cent of the force in H_2O-Ringer (while the tetanic force was reduced only by 15 per cent in average), the decline of the intensity of the active state was progressively enhanced in the course of the first 10—15 min. The time from stimulus to maximum force development remained unchanged (Fig. 5 B). The lowered intensity of the active state persisted as long as D_2O-Ringer was present and the muscle reversed to normal after a few minutes in H_2O-Ringer. In a number of experiments the decline of active state was determined after the resting force had been either decreased to zero or increased three times. The changes in the course of the decline were small and could not account for the difference observed between the two groups.

Discussion

The changes in the mechanical properties induced by replacement of water with deuterium oxide may be due to effects on some or all of the elementary processes involved in the contraction of the curarized muscle.

The amplitude of the *action potential* in turtle's muscle is not affected by deuterium (Goodall 1958) whereas the electroatriogram of frog's heart showed a 10 per cent decrease in amplitude and an increase in latency and in duration of the action potential (Kaminer 1960). In frog's semitendinosus the potential of the resting membrane and the peak of the action potential, as measured with intracellular electrodes, were unaffected by deuterium whereas the latency and the phase of repolarization increased to about twice their values in H_2O-Ringer (Buchthal and Engbæk, unpublished). These changes and also the substantial reduction in D_2O-Ringer of the frequency at which a complete tetanus could be maintained (Fig. 4) suggest that the primary excitatory processes are affected. On the other hand, there was no indication that the *conduction time* of the action potential was prolonged by deuterium since the changes in mechanical properties induced by deuterium were not abolished or reduced by using transverse instead of end-to-end stimulation.

The changes in active state accomplished by deuterium are probably due to effects on the excitatory processes (or on the excitation-contraction coupling). The decline of the active state was enhanced by deuterium in those muscles in which the twitch force was reduced by more than 50 per cent while it remained unchanged when the twitch force was reduced less. Since the changes were equally reversible after exchange of the D_2O-Ringer with H_2O-Ringer the different behavior can hardly be due to injury of some of the muscles. Neither could the difference be due to an incomplete exchange of hydrogen with deuterium as the decline in active state was the same a few minutes after and two to three hours after the application of D_2O-Ringer. A diminution of the duration of active state by chemical agents has hitherto been described only for monoiodo acetic acid (Aubert *et al.* 1957). There is no evidence for a true change in duration of maximum intensity of active state by deuterium since the increase observed can be explained in terms of lengthening of the latency period.

The increase in latency of the mechanical response in D_2O-Ringer is easily explained by the effect of deuterium on the latency of the action potential. The reduction of twitch force and rate of force development by deuterium may be caused *via* changes in the coupling between excitation and contraction and by a direct effect on the contractile substance. Goodall (1958) concluded from his experiments that the contractile substance was the site of the effect of deuterium since the amplitude of the action potential was not affected. He did not, however, take other changes of the excitatory processes or the excitation-contraction coupling into consideration. Kaminer (1960) suggested that the

excitation-contraction coupling is the link affected by deuterium and not the contractile substance since deuterium does not retard the shortening of the glycerol extracted rabbit's psoas as induced by addition of adenosine triphosphate. The slow diffusion of adenosine triphosphate in the model would, however, mask an effect on the rates of the reactions involved and the model experiments, therefore, do not exclude the possibility that the contractile proteins are affected by deuterium. That reaction rates rather than final states are affected is consistent with the present observation of an almost unchanged tetanic force even in experiments where the twitch force and the rate of force development were reduced to one fifth or less by deuterium.

I am greatly indebted to professor F. Buchthal, M. D. and to Mr. P. Rosenfalck, M. Sc. for valuable help and advice. The work was supported by grants from the Danish National Association against Rheumatic Diseases, Copenhagen, and the Muscular Dystrophy Associations of America, Inc., New York City.

Appendix

Inhibition of adenosine triphosphatase by deuterium oxide: Rabbit's psoas was extracted with 50 per cent glycerol at — 20° C for a period of two months. One g of the extracted muscle was homogenized in 5 ml of a salt solution (0.16 M KCl and 1 mM $MgCl_2$). One hundred μl of the homogenate were incubated with 0.85 ml 99.8 per cent deuterium oxide containing 0.16 M KCl and 1 mM $MgCl_2$ per liter or with the corresponding aqueous solution at 20° C for 90 min. 50 μl of 50 mM sodium adenosine triphosphate at pH 7.0 were added and the adenosine triphosphatase activity determined by electrometric titration at pH 7.0 and 20° C with 0.5 N NaOH. On the average the adenosine triphosphatase was inhibited by 45 per cent (Table I).

Table I.

Exp. no.	ATP-ase activity (μmoles/min per g muscle)	
	in H_2O	in 85—90 per cent D_2O
1	3.0	1.7
2	2.9	1.8
3	3.0	1.6
4	2.8	1.4

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The Loss of Added Lysine and Threonine During the Baking of Wheat Bread

By

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Abstract

Ericson, L.-E., S. Larsson and G. Lid. *The loss of added lysine and threonine during the baking of wheat bread.* Acta physiol. scand. 1961. *53.* 85—98. — Rat growth experiments and microbiological assay methods were used to estimate the loss of added free L-lysine·HCl and DL-threonine during the baking of wheat bread. Introductory rat experiments showed that the optimum concentration for growth of L-lysine·HCl added before baking was approximately 0.40—0.45 % of the fresh weight of the flour. The optimum concentrations of L-lysine·HCl and DL-threonine when added together were approximately 0.55 % for the former amino acid and 0.30 % for the latter. The loss of added L-lysine·HCl during baking was found to be 10—15 % as estimated by animal experiments and 5—10 % as estimated by microbiological assays. The loss of L-threonine (added as DL-threonine) was significantly greater, amounting to approximately 40 % as judged by the feeding trials and 20—25 % according to the microbiological estimations.

The amino acid compositions of the flour and the bread baked from the flour were also determined.

The addition of lysine to wheat bread results in a considerable increase in the protein value of the bread. A further improvement can be obtained by supplementation of the lysine-fortified bread with threonine (Hutchinson, Moran and Pace 1959; Ericson 1960). The addition of other single essential amino acids to wheat bread, either alone or in combination with lysine and threonine, does not seem to improve its protein value as judged by experiments with rats (Ericson 1960). Similar results have been obtained with wheat flour (Sure 1953, 1957, Deshpande, Harper and Elvehjem 1957).

In our previous experiments on the amino acid supplementation of bread, the amino acids were added to the bread *after* baking (ERICSON and OVENFORS 1959, ERICSON 1960). Numerous investigators have shown that lysine is easily inactivated in food by reactions with carbohydrates and other compounds. Less is, however, known about the stability of threonine. In the present study, an attempt was made to estimate the loss of added lysine and threonine during baking, using both microbiological and rat growth assays.

Experimental

Analytical procedures. For the specific determinations of the lysine and threonine contents of the breads and the diets, microbiological procedures were used. Lysine was determined turbidimetrically with *Leuconostoc mesenteroides* P-60 (ATCC 8042) using Difco's Bacto Lysine Assay Medium. The procedure followed that outlined by STEELE *et al.* (1949) with small modifications. Incubation, for instance, took place at 34° C for 24 h instead of at 37° C for 20 h as suggested by STEELE *et al.* This alteration of the incubation temperature was motivated by the publication of SCHIAFFINO, McGUIRE and LOY (1958) and was found to give a straighter standard curve and more reproducible results.

Threonine was determined with *Streptococcus faecalis* (ATCC 8043) using Difco's Bacto Threonine Assay Medium — again based on the findings of STEELE *et al.* (1949). The tubes were incubated at 37° C for 24 h.

The turbidity of the assay tubes after incubation was determined at 550 mμ in a Coleman Model 11 Universal spectrophotometer both when *L. mesenteroides* and *S. faecalis* were used.

The inoculum used in the assay of lysine with *L. mesenteroides* was obtained by cultivating the organism overnight at 37° C on Bacto Micro Inoculum Broth fortified as described by BOLINDER and LARSEN (1961). After centrifuging and washing the cells twice in sterile saline, they were suspended in a quantity of saline giving a Coleman transmittance reading of 50 %. Two ml of this suspension were added to 50 ml saline of which 2 drops were used to inoculate each assay tube.

A similar procedure was used for preparing the inoculum for *S. faecalis*, but the turbidity of the washed cells was in this case adjusted to a Coleman reading of 80. Of this cell suspension, a 2 to 50 dilution was made and 2 drops were used per assay tube.

Various conditions for the liberation of the lysine and threonine were investigated. It was noticed that more reproducible results were obtained when the samples (breads and diets) were extracted with ether : petroleum ether before being hydrolysed. Furthermore, hydrolysis in 3 N HCl gave a complete hydrolysis and also a lighter coloured hydrolysate and a smaller amount of insoluble material ("humin") than hydrolysis in 6 N HCl. For the microbiological determinations of lysine and threonine, the following procedure was therefore followed:

About 10 ml of a mixture of equal parts of ether and petroleum ether (Skellysolve F) was added to a known amount of sample, usually 0.4—0.6 g, in a 13 $\times$ 150 mm test tube. The tube was shaken carefully and centrifuged until a clear supernatant fluid was obtained. The solvent was sucked off through a capillary. The procedure was repeated three times. Residual solvent was finally evaporated off by placing the tubes in a stream of warm air.

Six ml of approximately 3 N HCl was added, care being taken to rinse the walls of the test tube free from sample and to let the acid moisten the entire content of the tube. The test tube was then sealed, and placed in a sand bath at 110° C. After 24 h, the tube was allowed to cool at + 4° C while still standing in the sand, a procedure which due to distillation results in a rinsing of the upper part of the tube. It was then opened and the content poured into a porcelain cup and evaporated to dryness on a water bath. The dark residue was suspended in a small volume of distilled water, filtered and washed. The filtrate was again evaporated to dryness, dissolved in distilled water, evaporated again and transferred with distilled water to a glass beaker. The pH was adjusted to 6.8 ± 0.2 with 0.5 and 0.01 N NaOH. From each sample treated in this way, three different dilutions calculated to fall on the standard curve, were prepared for the microbiological determinations. Each dilution, as well as each standard solution, was run in triplicate in these tests.

The more complete amino acid assays that were made on the flour and the non-supplemented bread were performed according to Moore and Stein (cf. SPACKMAN, STEIN and MOORE 1958). The Beckman/Spinco Model MS Amino Acid Analyzer was employed. These samples were hydrolysed in sealed evacuated ampoules in 6 N HCl at 110° C for 30—50 h, which was the routine procedure used by the group operating the apparatus. To 0.3 g of each sample, 10 ml of the acid was added. No attempt was made to find optimum hydrolysis conditions for the various amino acids. Tryptophan and cystine were not determined.

Nitrogen was determined by the Kjeldahl method as described by PERRIN (1953). The ammonia formed on distillation was collected in boric acid and titrated with 0.01 N HCl.

Dry weight determinations were carried out after heating the samples at 104—105° C for 24 h and letting them cool in a desiccator over silica gel.

Type of bread and baking conditions. The bread used in the present investigation was pan baked from a dough with the following composition:

water	1,000 g
wheat flour[1] (70 % extraction)	1,900 g
lard	20 g
roller-dried skim milk (< 1 % fat)	50 g
sugar	10 g
salt	20 g
malt extract	10 g
yeast	100—200 g

The amount of yeast was adjusted according to the total quantity of dough, more yeast being used when the quantity was small.

Dividing, rounding and moulding were done by hand before the dough pieces were put in the pans. Proofing took place at 45° C for approximately 25—50 min at a relative humidity of about 85 %. The baking temperature was 190—220° C and the bread was kept in the oven for about 20—30 min. Each bread loaf had a weight of approximately 400 g.

Before the bread was used for making the diets, it was cut into slices and dried in air at a temperature of 25—27° C for 2 days. It was thereafter ground in an Electrolux "Assistent" bread mill. The dry weight of the ground bread was 90—93 %.

When the amino acids were baked into the bread, they were dissolved in part of the water used for making the dough.

[1] Alexandra, Kungsörnen AB, Stockholm.

Table I. Dry weights, nitrogen contents and amounts of added L-lysine · HCl and DL-threonine for the various groups in Experiments I—III

Exp.	Group	Dry weight Bread %	Nitrogen content		Added	
			Bread[1] %	Diet[2] %	L-lysine · HCl[2] %	DL-threonine[2] %
I	A	90.9	2.00	1.88	—	—
	B	90.5	2.03	1.86	0.12	—
	C	92.1	2.02	1.90	0.25	—
	D	91.0	2.09	1.95	0.50	—
	E	91.4	2.10	2.00	0.80	—
II	A	93.0	2.18	1.97	0.50	—
	B	92.9	2.19	1.99	0.50	0.30
	C	93.0	2.25	2.01	0.80	0.08
	D	92.6	2.26	2.06	0.80	0.15
	E	92.6	2.26	2.07	0.80	0.30
III	A	92.3	2.19	2.03	[3]0.30	—
	B	92.3	2.19	2.04	[3]0.40	—
	C	92.6	2.24	2.03	0.40	—
	D	91.4	2.27	2.08	0.50	[3]0.15
	E	91.4	2.27	2.12	0.50	[3]0.25
	F	91.5	2.26	2.12	0.50	0.25

[1] Expressed as per cent of the weights of air-dried bread.

[2] Expressed as per cent of the fresh weights of the diets.

[3] These amounts of L-lysine · HCl or DL-threonine were added to the diets *after* baking. In all other cases the amino acids were incorporated into the dough.

The diets. All diets contained 91 % of ground bread, 3 % of a salt mixture, 5 % soya bean oil, 0.5 % cod liver oil and a vitamin mixture. The vitamins were mixed with a small quantity of finely powdered bread before they were added to the general mix.

The salt mixture was identical with that of HEGSTED *et al.* (1941) and the vitamin mixture had the composition given by HARPER *et al.* (1953). The amounts of the different vitamins in mg per 100 g of ration were: thiamin · HCl 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, pteroylglutamic acid 0.02, cyanocobalamin 0.002, inositol 10, menadione 0.5, and choline chloride 150. The cod liver oil supplied approximately 375 I. U. vitamin A and 37.5 I. U. vitamin D per 100 g of ration. These values are higher than those in our previous experiments (ERICSON and OVENFORS 1959, ERICSON 1960) which is motivated by the longer duration of some of the experiments in the present study.

Conditions of the rat experiments. The rats used in the present investigation were males of the Sprague-Dawley strain, having an initial weight of 40—60 g. They were housed individually in cylindrical glass vessels with a 20 cm diameter containing a layer of wood shavings. The temperature in the animal room was 27° C. The animals were permitted to consume the diets *ad libitum.* They were weighed twice a week. With the exception of the first experiment, the rats were fed a non-supplemented bread diet

Table II. Amino acid composition of the wheat flour and the non-supplemented bread baked from the flour and used in the present investigation. Determination according to Moore and Stein after hydrolysis in 6N HCl for 30 h

Amino acid	Wheat flour[1] g/16 g N	Wheat bread[2] g/16 g N
Alanine	2.76	2.52
Arginine	3.18	3.14
Aspartic Acid	4.37	4.43
Cystine	—	—
Glutamic acid	36.0	30.8
Glycine	3.40	3.14
Histidin	1.64	1.90
Leucine	6.89	6.45
Isoleucine	3.94	3.72
Lysine	1.93	2.60
Metionine	1.34	1.31
Phenylalanine	4.80	4.48
Proline	12.1	10.4
Serine	3.73	3.85
Threonine	2.30	2.57
Tryptophan	—	—
Tyrosine	2.50	2.50
Valine	4.48	4.36

[1] Nitrogen content 1.82 %, dry weight 85.7 %.
[2] Nitrogen content 2.23 %, dry weight 89.5 %.

for a period of 5—7 days before they were given the various experimental diets. This was done in order to accustom the rats to bread diets.

Table I summarizes the dry weights, nitrogen contents and amounts of added L-lysine·HCl (Pfizer & Co, Inc.) and DL-threonine (Fluka, A. G.) for the various groups of diets used in the three different rat experiments. The figures given for L-lysine·HCl and DL-threonine are approximate and represent the amounts calculated before the baking was done on the basis of the weight of air-dried bread obtained from a certain weight of wheat flour. The baking of the bread for these experiments as well as for Experiment IV (see Table III and IV) was done on separate occasions.

Results

Amino acid determinations. The amino acid compositions of the wheat flour and the non-supplemented bread are given in Table II. The values are expressed as g per 16 g of nitrogen according to practice, although this is not entirely correct for cereal products. There are only rather minor differences between the amino acid compositions of flour and bread, the more noticeable ones being the changes in glutamic acid and lysine. The increase in lysine is due to the inclusion of roller-dried skim milk and yeast in the bread.

Table III. Recovery of added L-lysine·HCl from bread and diets as determined by microbiological assays. For details of the calculations see text

Exp.	Group	Experimentally determined L-lysine·HCl in per cent of the dry weight of the		Calculated content of L-lysine·HCl in per cent of the theoretical dry weight of the		Re-covery %
		Bread	Diet	Bread	Diet	
I	D	0.45		0.59		80
	D		0.53		0.53	100
II	A	0.45		0.60		75
	A		0.43		0.54	80
	B	0.52		0.60		87
	B		0.42		0.54	78
III	C	0.41		0.48		85
	C		0.40		0.43	93
	E	0.48		0.60		80
	E		0.45		0.54	83
	F	0.50		0.60		83
	F		0.46		0.54	85
[1]IV		0.54		0.60		90
			0.48		0.54	89
						Average 84.8 ± 1.7[2]

[1] No corresponding rat experiment reported here.
[2] Standard error of the mean.

Table IV. Recovery of added L-threonine from bread and diets as determined by microbiological assays. For details of the calculations see text

Exp.	Group	Experimentally determined L-threonine in per cent of the dry weight of the		Calculated content of L-threonine in per cent of the theoretical dry weight of the		Re-covery %
		Bread	Diet	Bread	Diet	
II	B	0.10		0.18		56
	B		0.09		0.15	60
III	F		0.06		0.135	45
[1]IV		0.07		0.12		58
			0.07		0.11	64
						Average 56.6 ± 3.2[2]

[1] No corresponding rat experiment reported here.
[2] Standard error of the mean.

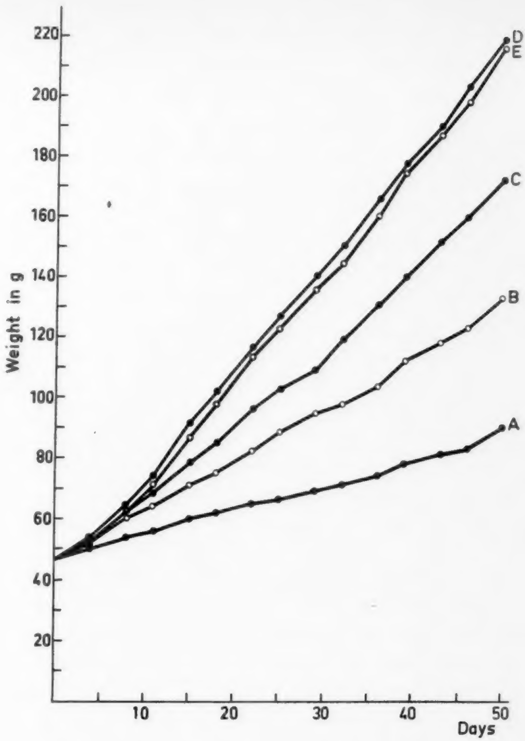

Fig. 1. Growth curves for the rats in Experiment I.

Curve A: Non-supplemented bread diet.

Curve B: Bread diet containing 0.12 % added L-lysine·HCl.

Curve C: Bread diet containing 0.25 % added L-lysine·HCl.

Curve D: Bread diet containing 0.50 % added L-lysine·HCl.

Curve E: Bread diet containing 0.80 % added L-lysine·HCl.

The L-lysine·HCl was incorporated into the dough.

Before presenting the data on the loss of free lysine and threonine during baking, as estimated by microbiological methods, it is necessary to discuss briefly how the calculations were performed that led to the figures shown in Table III and IV. Firstly, it was assumed that the flour for the dough was accurately weighed and used without loss. Secondly, the dry weight of the flour was assumed to be the same (86 %) on all occasions although dry weight determinations were not made in each case. Thirdly, a loss of 4 % due to carbon dioxide, alcohol, etc. was calculated to take place during the fermentation, proofing and baking of the bread. Taking into account the amount of dry matter supplied by the other ingredients of the dough, one finds that 1,900 g flour should theoretically give 1,720 g of dry bread.

Since known amounts of L-lysine·HCl or DL-threonine were added per unit weight of flour it is possible to express the amount of added amino acid as per cent of the theoretical dry weight of the bread. Knowing the amount of air-dried bread in the diets and the dry matter content of this bread, one

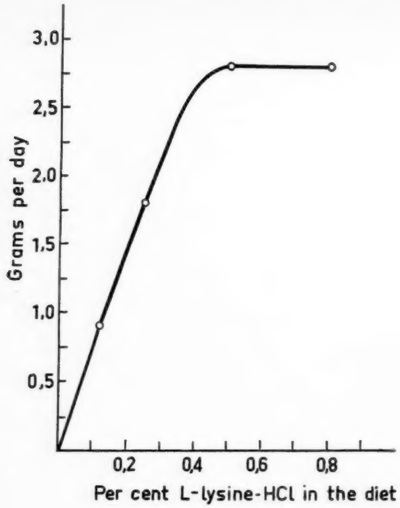

Fig. 2. Growth rate for the rats in Experiment I in g/day above that obtained with the non-supplemented bread diet as a function of the amount of added L-lysine·HCl expressed as per cent of the fresh weights of the diets

can also calculate the per cent of added lysine and threonine on the basis of the theoretical dry weight of the diet. Determinations of lysine, threonine and dry matter were done for both breads and diets and a recovery value was calculated as shown in the tables. This recovery value is expressed as the content found experimentally divided by the theoretical content, calculated as outlined above, and multiplied by 100.

It should be mentioned that the data in Table III and IV represent only a part of all the estimations done. Only data from experiments with such levels of added lysine and threonine that were considered of practical importance (see under "Rat experiments") are presented. It should also be pointed out that the recovery as given in the tables comprises not only the loss during baking (including fermentation and proofing) but also during the drying of the bread and the hydrolysis of the samples preceding the amino acid assays.

The loss of lysine estimated by microbiological methods and expressed as just outlined is illustrated by the values in Table III. The loss of threonine is shown in Table IV. The values will be discussed further (see under "Discussion") after that the results from the rat experiments have been presented.

Rat experiments. In the first series of rat experiments, 5 different groups of rats were used, initially comprising 12 animals each. The first group received a non-supplemented bread diet and the four subsequent groups diets containing breads baked from doughs to which various amounts of L-lysine·HCl had been added. The levels of L-lysine·HCl added corresponded to approximately 0.12, 0.25, 0.50 and 0.80 % of the fresh weights of the diets.

The growth curves are shown in Fig. 1. It can be seen that the growth rate increased with increasing concentrations of lysine up to the level of 0.50 %. Above this concentration, no improvement in growth was observable. This is more easily seen from Fig. 2 where the increase in growth rate in grams per day above that of the group receiving the non-supplemented bread diet is plotted against the concentration of added L-lysine · HCl expressed as per cent of the fresh weights of the diets. Fig. 2 shows that a nearly linear relationship exists between the growth rate and level of added lysine as long as the concentration of the L-lysine · HCl is less than approximately 0.35 %. Previous experiments in which the L-lysine · HCl was added after baking (Ericson and Ovenfors 1959) had demonstrated that no improvement in growth rate was obtained when the level of L-lysine · HCl was increased above 0.40 % of the diet weight. This, together with the significant effect observed in the present experiment even with the lowest concentration of lysine incorporated into the dough, suggested that the inactivation or destruction of lysine during baking was comparatively small.

If the growth rates are calculated from the eighth day onwards, when the growth curves in Fig. 1 are nearly linear, the following values are obtained for the different groups (mean and S. E.): A 0.78 ± 0.03, B 1.67 ± 0.04, C 2.57 ± 0.06, D 3.59 ± 0.02 and E 3.57 ± 0.05.

Six of the animals in each group were killed after 22 days and the remaining after 50 days. On all of the rats, determination of body fat and liver fat as well as histological examinations on the livers were carried out. The results of these observations together with those on rats from a number of other experiments — including Experiments II—IV — will be presented in a separate paper (Larson, Rubarth and Ericson 1962).

In the next series of experiments various combinations of lysine and threonine were added to the dough in order to gain information on the optimum concentrations of these amino acids when they are incorporated together before baking as well as the degree of destruction or inactivation of the threonine. Five groups of rats, each comprising 10 animals, were given the diets during 36 days. The levels of L-lysine · HCl and DL-threonine incorporated — expressed in per cent of the weights of the diets — are given in Table V together with the results. It is obvious that the addition of threonine to a dough already containing lysine results in a significant improvement of the nutritive value of the bread. This is in accordance with observations made previously that a combination of both lysine and threonine is superior to lysine alone when the amino acids are added to bread diets after baking (Ericson 1960). The combination of 0.50 % L-lysine · HCl and 0.30 % DL-threonine was also found to be almost as effective as 0.80 % L-lysine · HCl and 0.30 % DL-threonine (Table V). A plot of gain of weight above that of the control group versus added DL-threonine indicated that the maximum effect had not been obtained at the highest level (0.30 %) tested in this series.

Table V. Composition of diets and growth rates for the five groups of rats in Experiment II. Ten rats were used per group. The amino acids were added before baking

Group	Diet	Growth rate g/day
A	Basal + 0.50 % L-lysine·HCl	3.96 ± 0.09[1]
B	As A + 0.30 % DL-threonine	6.05 ± 0.09
C	Basal + 0.80 % L-lysine·HCl + 0.08 % DL-threonine	5.03 ± 0.03
D	As C but with 0.15 % DL-threonine	5.68 ± 0.05
E	As C but with 0.30 % DL-threonine	6.36 ± 0.15

[1] Standard error of the mean.

Table VI. Composition of diets and growth rates for the six groups of rats in Experiment III. Twelve rats were used per group

Group	Diet	Growth rate g/day
A	Basal + 0.30 % L-lysine·HCl added *after* baking	3.59 ± 0.10[1]
B	Basal + 0.40 % L-lysine·HCl added *after* baking	4.03 ± 0.11
C	Basal + 0.40 % L-lysine·HCl added *before* baking	3.75 ± 0.02
D	Basal + 0.50 % L-lysine·HCl added before baking + 0.15 % DL-threonine added *after* baking	4.97 ± 0.05
E	Basal + 0.50 % L-lysine·HCl added before baking + 0.25 % DL-threonine added *after* baking	5.15 ± 0.05
F	Basal + 0.50 % L-lysine·HCl added before baking + 0.25 % DL-threonine added *before* baking	4.92 ± 0.04

[1] Standard error of the mean.

In the third series, a direct comparison between the effects of bread diets to which lysine or lysine plus threonine had been added before or after baking was attempted. When lysine alone was studied, the concentrations of lysine added after baking were 0.30 and 0.40 % L-lysine·HCl while the concentration added before baking was set at 0.40 %, again expressed in per cent of the fresh weights of the diets. These levels were considered appropriate since they were close to the optimum conditions for supplementation of bread with lysine alone (Exp. I). The levels of DL-threonine added after baking were 0.15 and 0.25 % and the amount added before baking 0.25 % of the diet weights. In these cases, we increased the amount of L-lysine·HCl added to 0.50 %, as Exp. II had indicated this to be necessary in order to obtain full effect from the threonine supplementation.

The results are shown in Table VI. It can be seen that 0.40 % L-lysine · HCl added before baking does not give the same growth-promoting effect as 0.40 % added after baking, but that it is better than 0.30 % added after baking. The data thus indicate that the inactivation of lysine during baking of wheat bread is comparatively insignificant. In the case of threonine, the picture is different. The level of DL-threonine added before baking, *viz.* 0.25 %, did not quite give the same growth rate as the lowest level, *viz.* 0.15 %, added after baking. This suggests a rather large loss of added threonine during the baking of bread.

Discussion

There is a large number of reports on the availability and heat inactivation of naturally occurring lysine in a variety of food items. These studies indicate that protein-bound lysine can be made nutritionally unavailable by at least two different types of reactions: (a) by reactions with other amino acids giving "unnatural" peptide linkages between the ε-amino group of lysine and a carboxyl group of another amino acid which are resistant to enzymic digestion and (b) by reactions between the ε-amino group of lysine and carbohydrates (or other compounds containing aldehyde groups *e. g.* gossypol) which finally leads to the formation of brown, humin-like condensation products and which again makes the lysine biologically unvailable. From the first type of reaction product, the lysine can be released by acid hydrolysis. In the latter case, this is possible only at the initial stages of the interaction — if the heat damage is too severe it becomes impossible to recover the lysine. A third possible cause of loss — the direct monomolecular destruction of the lysine — is generally not considered to be of importance in the preparation of food for consumption.

Attempts have also been made to determine the loss of free lysine during the preparation of food, in particular during the baking of wheat bread fortified with DL- or L-lysine · HCl. Rosenberg and Rhodenburg (1951) studied this problem using microbiological assay methods. They concluded that when 0.25 % L-lysine · HCl was added to the flour, about 32 % was lost during the baking. However, in a subsequent paper (Rosenberg and Rhodenburg 1952), the same authors used rat experiments to investigate the loss and then found that an equal response was obtained from lysine added to flour before baking or from lysine added in corresponding amounts to the bread diet. Sabiston and Kennedy (1957) arrived at a similar conclusion in their comprehensive feeding experiments. Their results showed no difference in protein efficiency ratios between groups of rats fed diets containing bread to which the lysine had been added before or after baking. Cremer *et al.* (1951), on the other hand, performing nitrogen balance studies on human subjects, reported an almost complete loss of free DL-lysine added to wheat bread before baking.

The loss of added L-lysine · HCl in baking powder biscuits has been studied with microbiological methods by CLARK *et al.* (1959) and was found to be less than 10 %.

Our own animal experiments indicate that 85—90 % of the free L-lysine · HCl added to the dough is present in a biologically available form in the bread. The microbiological assays gave recovery values for breads and diets ranging from 75 to 100 % (average about 85 %). It should once again be pointed out that this figure represents the recovery of the lysine not only from the baking process but also from the drying of the bread and the hydrolyses of the samples.

Judging from the observations of SABISTON and KENNEDY (1957) and of ROSENBERG *et al.* (1951, 1958), it seems unlikely that the drying procedure used here would have caused any significant loss of added lysine. The hydrolysis and accompanying operations, on the other hand, might well give rise to some loss and thereby lower the recovery values. This possibility was tested by adding a known amount of L-lysine · HCl to a bread sample and submitting the mixture to hydrolysis under the conditions described earlier. The recovery was 92 %. (Adding the same amount of L-lysine · HCl to the bread after hydrolysis resulted in a 100 % recovery.) Assuming on the basis of this observation a 5—10 % loss during the hydrolysis and subsequent operations, one arrives at a recovery figure for the loss of free L-lysine · HCl during baking alone of 90—95 %. This figure agrees well with that obtained from the rat experiments keeping in mind that acid hydrolysis often releases more lysine than enzymic processes.

There seems to be no data published on the loss of free added threonine during the baking of wheat bread. Although it is known that some loss of protein-bound threonine can take place during the heating of carbohydrate-rich foods, we did not expect to find the loss of free threonine, as judged by both rat experiments and microbiological estimations, to be considerably greater than that of lysine. The former type of assay indicated that only about 60 % of the DL-threonine added to the dough was biologically available in the bread. The microbiological determinations gave recovery values ranging from 45—64 % with an average value of about 57 %. The loss of threonine during the hydrolysis was found to be rather large, approximately 25—30 %. (Adding the threonine to bread after hydrolysis resulted in a 100 % recovery.) Taking these latter figures into account, one finds that the recovery of threonine from the baking and drying processes as estimated by microbiological methods would be about 75—80 %. The reason for the discrepancy between the rat assay and the microbiological assay is not known, but the data suggest that free threonine can be made biologically unavailable to higher animals by reactions with components in food to compounds from which it can, however, be partially released by acid hydrolysis. Further studies are necessary to clarify this point. It is conceivable that the potassium bromate present in the flour (30—40 mg/kg) was partly responsible for the loss of free threonine.

A direct comparison between the amino acid composition of the wheat flour and bread used in this investigation and literature data has not been possible as, to the author's knowledge, such determinations have not previously been made on Swedish flour and bread. However, SIHLBOM (1961) has recently carried out a study on the amino acid composition of a number of varieties of Swedish wheats and also on one sample of wheat flour that contained, after extraction of lipids, 1.67 % nitrogen. The values obtained are in good agreement with those reported here, possibly with the exception of histidine, leucine and serine which showed slightly higher values in the analyses of SIHLBOM.

The baking properties of the type of wheat bread investigated in this study was not significantly altered by the addition of the amount of L-lysine · HCl necessary for optimum improvement of the nutritive value (about 0.40—0.45 % L-lysine · HCl based on the fresh weight of the flour). Only a slightly darker crust was noticed in most cases. No changes in taste were observed but properly planned tests to detect any differences were not performed. Doubling the lysine addition, however, gave a much darker, and also considerably more compact loaf of bread. The addition of a few tenths of a per cent of DL-threonine to lysine-fortified bread was considered by some of us to give an undesirable flavour to the crust. Again it should be emphasized that this observation was wholly subjective.

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